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THE LIVING UNIVERSE

An Encyclopaedia of
the Biological Sciences

The world of microbes

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THE LIVING UNIVERSE : AN ENCYCLOPAEDIA OF THE BIOLOGICAL SCIENCES

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An Encyclopaedia of the Biological Sciences

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The world of microbes

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NELSON

Ever since Pasteur isolated the first bacillus in 1857, detailed research has shown that our existence is ultimately related to that of these microscopic forms of life, of which there are millions of different kinds. Even the smallest drop of water contains thousands of millions of individual microbes. Is life conceivable without microbes? Certain biologists are now trying to prove that it is; and it was for this purpose that Reyniers' isolator, depicted here, was designed. Within the steel cylinder provided with observation portholes, animals have lived and reproduced their kind without any microbial contamination. Will this experiment one day become a commonplace? Whether it will or not, we are living at present in a world of microbes, and the following pages provide a wide and precise survey of this microscopic world.

THE LIVING UNIVERSE

An Encyclopaedia of the Biological Sciences

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The world of microbes

This volume of THE LIVING UNIVERSE, *an Encyclopaedia of the Biological Sciences, has been produced under the direction of* Albert DELAUNAY *and* Romolo DEOTTO, *with the collaboration of* Carlo ARNAUDI, *Director of the Technical Institute of Soil Microbiology at Milan University;* Luigi CALIFANO, *Director of the Institute of General Pathology at Naples University;* Giorgio CAVALLO, *Professor of Microbiology at Sassari University, Italy;* Léon DMOCHOWSKI, *M.D., Ch.B., Ph.D., Head of the Department of Virology and Electron Microscopy at Texas University, USA;* Robert FASQUELLE, *Professor of Microbiology at the Faculty of Medicine, Paris;* Charles GERNEZ-RIEUX, *Director of the Pasteur Institute and Member of the National Academy of Medicine, Paris;* Claude HANNOUN, *D.Sc., Head of the Pasteur Institute Laboratory, Paris;* Henry HARRIS, *Head of the Department of Cellular Biology at the John Innes Institute, Herts, UK;* Paul HAUDUROY, *Professor at the Faculty of Medicine, Lausanne and Paris;* William E. VAN HEYNINGEN, *D.Sc., Cambridge, D.Sc., Oxford;* Nikolaï D. IEROUSSALIMSKY, *D.Sc. (Biology), Professor at Moscow University and Corresponding Member of the USSR Academy of Sciences, Moscow;* Alick ISAACS, *M.D., of the National Institute of Medical Research, Mill Hill, London;* Holger W. JANNASCH *of the Institute of Microbiology at Göttingen University, Germany;* Serghiei I. KOUZNETSOV, *Professor at the Institute of Microbiology, USSR Academy of Sciences, Moscow;* Giulio A. MACCACARO, *Assistant at the Institute of Microbiology, Milan University;* Paul MANDEL, *Professor at the Faculty of Medicine, Strasburg;* J. G. MARCHAL, *D.Sc. (Natural Sciences), Professor of Microbiology at the Faculty of Pharmacy, Nancy, France;* Pierre NICOLLE, *M.D., Department Head at the Pasteur Institute, Paris;* James A. REYNIERS, *M.Sc., LL.D., Director of the Aseptic Life Research Centre, Tampa, USA;* Hans SCHMIDT, *M.D., Emeritus Professor of Hygiene at Marburg University, Germany;* Wayne W. UMBREIT, *Professor of Bacteriology at Rutgers University, New Brunswick, NJ, USA. Introduction by* Albert DELAUNAY.

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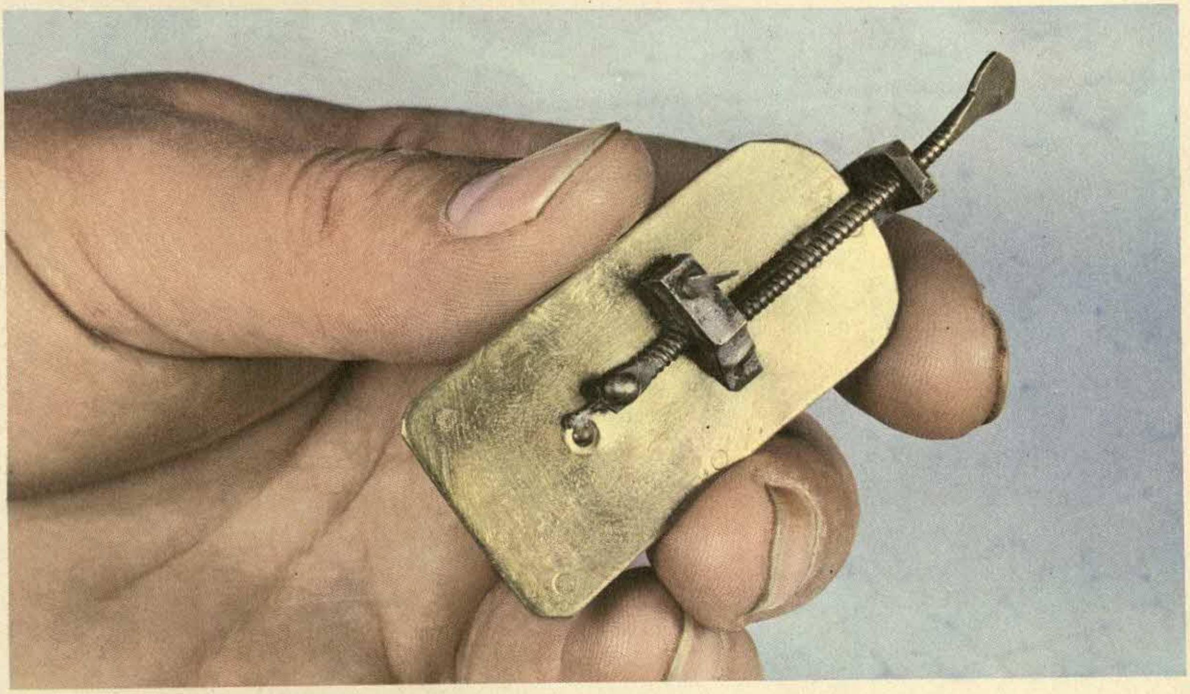
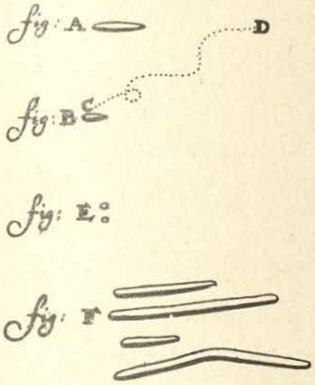
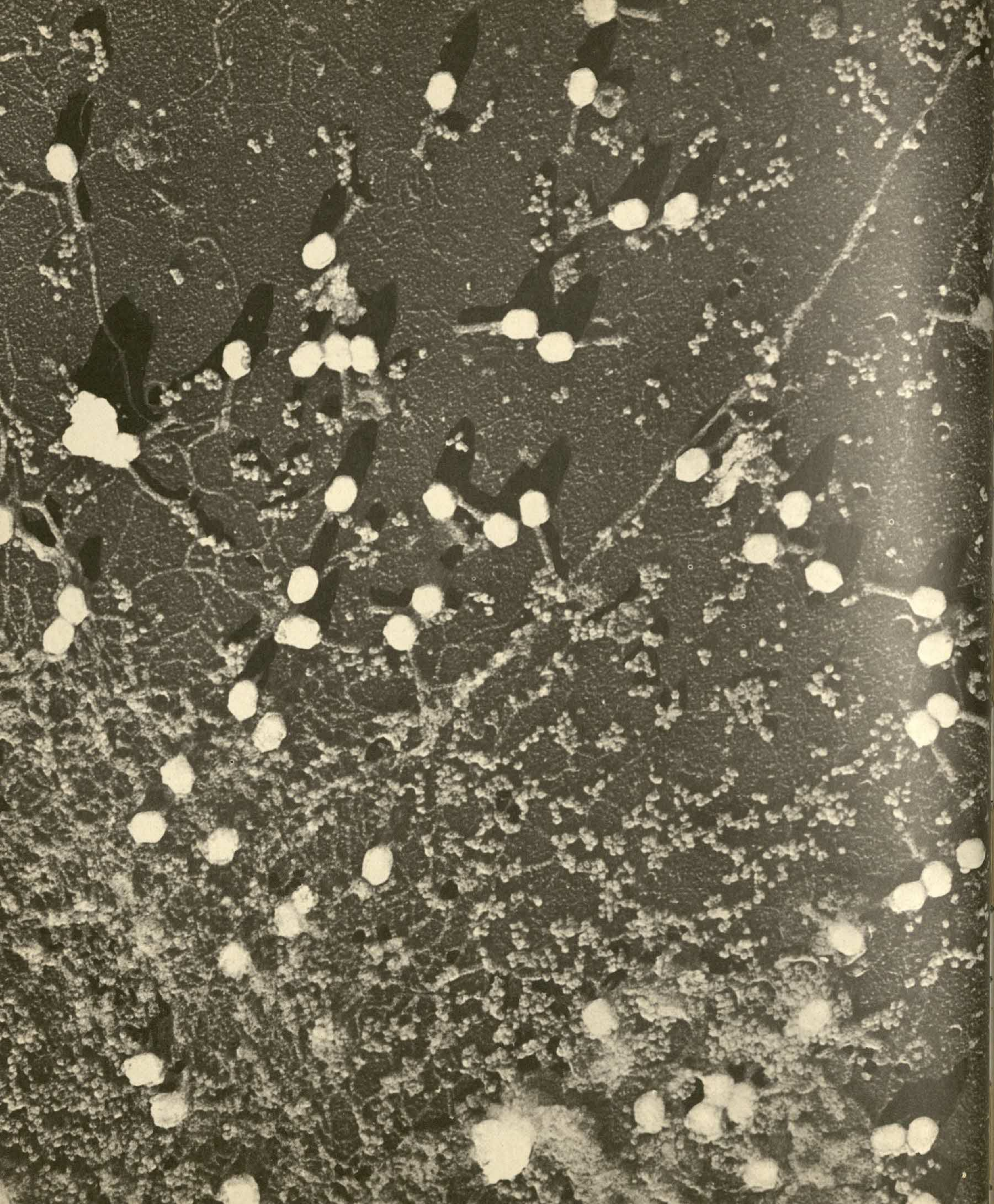


A plate of brass with a small spherical lens and fitted with a fine pointed shaft for holding an object near to the lens—such was the microscope constructed by van Leeuwenhoek. (Photo Lod).



THE great lesson gained from the observations of van Leeuwenhoek is that on all sides, so to speak, wherever enlarging vision is directed, one sees the examined object come alive. The universe is much more inhabited than once we believed: rest, inertia, is merely an illusion due to the inadequacy of our senses. A drop of stagnant water becomes an ocean in which an entire population swims. A piece of dental tartar houses more living creatures than there are human beings on earth. And these small beings, these animalculae, these animated atoms revealed by the microscope, are of all sizes and shapes—vesicles, rods, spirals. All is restless, alive, and in a state of constant agitation. Everywhere there is life and movement. The day that Leeuwenhoek discovered these microbes, he exclaimed: 'Of all the wonders that I have discovered in nature, this is the greatest. . . . And I must say that I have never known so pleasant a sight as that of thousands of these small creatures living in a drop of water, stirring as one group, yet each having its own movement.'

JEAN ROSTAND



The world of microbes

Charles GERNEZ-RIEUX

It was truly a new world that was discovered nearly three centuries ago by Anton van Leeuwenhoek, whose name marks the birth of bacteriology. This modest Council Chamber usher from Delft used to pass his spare time in examining the most diverse objects through lenses which he had ground himself. He possessed hundreds of these lenses, of rock crystal or even diamond, and with them he was able to observe in a drop of rain-water minute 'animalculae' which gambolled about 'like a cloud of gnats', so numerous that when he first saw them he believed himself to be suffering from hallucinations.

Encouraged in these observations by his young compatriot Regnier de Graaf, who introduced him to the Royal Society of London, van Leeuwenhoek addressed nearly 400 scientific papers to the Society between the years 1676 and 1719. That of 17th September 1683 might well be claimed to have founded bacteriology, for in it we find a perfect and detailed description, accompanied by remarkable diagrams, of the main types of microbe: cocci, rods and spirals, so small that one 'grain of sand could hold more than a million', and so numerous that on a single human tooth Leeuwenhoek counted more microbes 'than there are subjects in a kingdom'.

The four volumes of *Arcana naturae ope et beneficio exquisitissimorum microscopiorum detecta*, published in 1695, opened a new era in science by making known the world of the infinitely small.

The reputation of van Leeuwenhoek was considerable: he was visited by Charles II and George I, and by the Czar of Russia, Peter the Great. But unfortunately the minds of the period were not yet ready to understand the full implications of his discovery. Newton had formulated the law of universal gravity, Leibnitz the principles of the differential calculus, and Fermat the theory of numbers: this essentially rational century, stimulated by Descartes, was primarily orientated towards mathematics and physics. It had not yet opened its eyes to biology. Several years before van Leeuwenhoek discovered the world of microbes, the chemist van Helmont had given his prescription for the rearing of mice: 'Put some soiled cloth into a jar full of wheat; towards the twenty-first day fermentation ceases and, as a result of the emanations from the grain, true mice will be born in the jar. These are capable of mating with normal animals'.

The observations of van Leeuwenhoek could create no stir in the scientific world of his time. Their sole effect was to cause the Swedish botanist Linnaeus to create a special class of living beings, the *Chaos*: the 'animalculae' were placed in the species *Chaos infusorium*, where they long remained together with the spermatozoa and the 'airy clouds in blossom time'.

Although the discovery of micro-organisms aroused a certain academic interest, it was not until two centuries later that we came to understand the extraordinary role that they play in the world.

The work of Pasteur

The credit for this development must go to Louis Pasteur and it is therefore worth while recalling the background of his work. In 1854, he had been nominated Head of the Faculty of Sciences at Lille and, preceded by the reputation he had acquired by his studies in molecular asymmetry, this professor of chemistry decided to devote part of his time to the local industries. As a result of this new orientation he received a visit from the father of one of his students, a man named Bigo who was an industrialist at Lille. Bigo, who was experiencing serious disappointment in the manufacture of alcohol from sugar-beet, asked for advice. The prospect of enlarging the sphere of his teachings by undertaking new researches incited Pasteur to agree to investigate the problem at the Bigo factory. With his simple microscope he was able to observe that the globules in the fermenting

The study of bacteria under the electron microscope occasionally reveals astonishing facts. Seen here (opposite page) is a ruptured (or lysed) bacterium full of bacteriophages which have reached maturity (white blobs with a tail). Amongst the cellular debris are seen some fibres of unused DNA. *(Photo: Laboratory of Biophysics, University of Geneva).*

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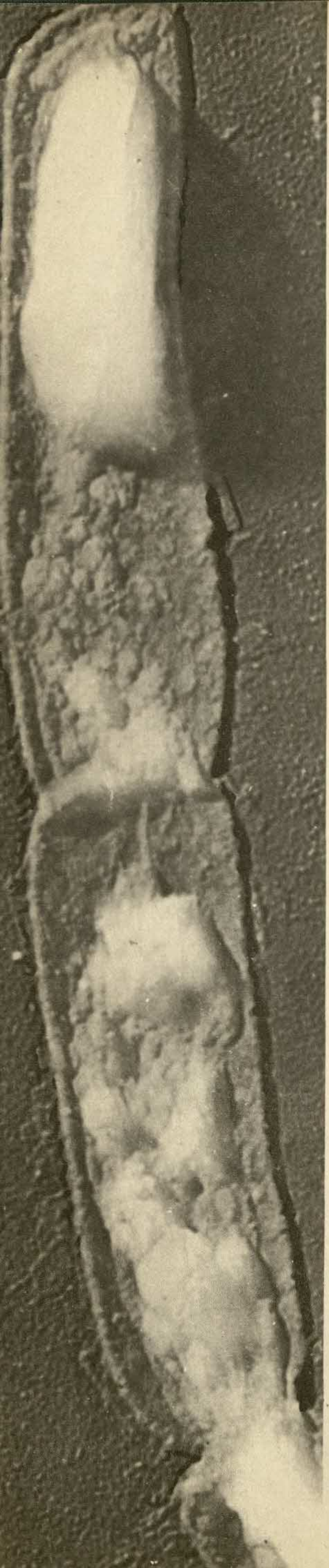
(1) *At least, this is the opinion of a fairly large number of researchers; but other theories have also been put forward.*

Bacteriologists divide the micro-organisms they study into two main groups—pathogenic or parasitic germs and non-pathogenic or saprophytic germs. The latter can live at the expense of inert or organic matter whether animal or vegetable in origin. In nature the saprophytic germs are found in many different sites and in infinite variety, as for example this mildew on elm bark which has slowly rotted away in a pool. (Photo Lod).



juice were round when the fermentation was normal; but if something had gone wrong, he found that side by side with the normal globules there were other small entities, elongated in form, which had until then escaped attention. He was able to isolate this ferment and cultivate it in the appropriate liquid, thus reproducing the unwelcome lactic fermentation which was the whole source of the trouble.

Although the researches of chemists from Liebig to Berzelius had led them to reject the idea of any influence whatever of organization and of life on the process of fermentation, Pasteur affirmed that the yeast he had discovered was the specific living cause of fermentation, and on 3rd August 1857 he presented before the Society of Sciences of Lille his famous *Mémoire sur la fermentation appelée lactique*, which in a mere fifteen pages indelibly moulded the new direction of his scientific thought.

The whole of Pasteurian doctrine is enshrined in this memoir in which we find a clear exposition of the foundations of bacteriological technique, notably the idea of isolating germs and the necessity of selecting a suitable culture medium. Pasteur even urged the use of antibiotics.

But what dominates his paper is the general viewpoint which was destined to guide scientific thought: 'I have reasoned,' he wrote, 'within the hypothesis that the new yeast is organized, that it is a living being and that its chemical action on the sugar is related to its development and organization. . . . It seems to me from my own knowledge of the subject, that anyone who cares to judge the results of this work impartially, together with those I shall publish next, will agree that fermentation is correlated with the life and the organization of the globules.'

The memoir of 1857 marked a decisive turning-point in the history of biology. For the first time the idea was clearly put forward that the transformation of organic matter is effected by the intervention of these living micro-organisms, the study of which had previously been the prerogative of botanists. In showing that each microbial type is characterized by a specific chemical reaction and develops only in highly selective conditions, Pasteur had given us the means of orientating the organic processes according to the needs and wishes of man at grips with nature.

In adopting the biological theory of fermentation, he not only revealed microbial life, but made it possible for us to control it.

Bacteria and the equilibrium of life

The path travelled since then has been long, and the history of the study of these microscopic organisms, to which C. Sedillot in 1878 gave the name of microbes, is a truly remarkable one.

Offsetting the extreme smallness of their size by a power to multiply and a biochemical activity that are truly amazing, microbes—and more particularly the bacteria—play an essential role in the equilibrium of life on the earth's surface. Some, called autotrophic, build up their protoplasm fully, starting from purely mineral substances. As for the energy they need, they borrow it either from chemical reactions between inorganic substances (chemosynthetic bacteria), or from light (photosynthetic bacteria). The former are widespread, in soil and in water. For example, the bacterium *Nitrosomonas* oxidizes ammonia into nitrous acid, whilst *Nitrobacter* transforms the nitrous acid into nitric acid. These two types of bacteria are responsible for the important phenomenon of nitrification of soils which plays a major role in agriculture.

The photosynthetic bacteria contain a green pigment, bacterio-chlorophyll, often masked by red carotenoid pigments (purple bacteria). Also widespread in soil and in water, these curious bacteria are capable of utilizing hydrogen sulphide. Together with the other sulphur-bacteria, they break down this gas and the evil-smelling and poisonous sulphides, and transform them into sulphites or sulphates which can be utilized by chlorophyll-containing plants. Here lies their importance.

In nature, most organic substances are rapidly destroyed by bacteria but a few are particularly resistant to their action. Typical of the latter is lignin which, with cellulose, is one of the principal constituents of the woody parts of plants. In the soil we come across all the intermediate products of the gradual transformation of these substances. The most complex constitute humus, accumulations of which give rise to peat and can become fossilized as coal. Asphalt and petrols are probably also of a bacterial origin.

The life of bacteria in soil is, in other respects, closely linked with the growth of

In the course of the terminal phase of bacterial division a partition is formed which divides the mother cell into two daughter cells. Such phenomena have only recently been revealed. (Mag. × 21,000. Istituto Superiore di Sanità, Rome).

plants. Certain bacteria, the *Rhizobia*, live in symbiosis with leguminous plants. They can be found in the nodules on the roots of these plants and cause the synthesis of nitrogenous compounds from gaseous nitrogen which the host plant can then use for its own growth. Other bacteria directly fix the nitrogen of the air, and also play an important role in the fertilization of the soil.

The bacteria which live in proximity to the roots of crops influence the growth of these plants. The rhizosphere, that part of the soil situated in the immediate neighbourhood of the root, is particularly rich in micro-organisms which elaborate the mineral substances capable of being assimilated by the plant. These bacteria can even form close casings around rootlets, causing separation from their environment and interference with nutrition.

The role of bacteria is no less important in the processes involving the destruction of dead animals and plants. Without their intervention the amount of unusable organic matter would continue to increase upon the soil and in the water. Proteins are decomposed into polypeptides, then into amino acids, which in their turn are transformed by bacterial action into ammonia, whilst the carbonaceous residue from the breakdown is ultimately destined to end up as carbon dioxide and water, through combustion.

Thus the whole of the organic nitrogen of more complex organisms is transformed into ammonia by the work of bacteria. But as we have already seen, the autotrophic bacteria intervene to convert this nitrogen once more into substances which can be utilized by green plants, both terrestrial and aquatic, and so the nitrogen cycle is completed.

It is remarkable that it should be the smallest of living beings that are allotted a major role in the cyclic circulation of atoms of carbon, nitrogen and sulphur over the face of the earth. According to Boivin, there are good reasons for assuming that they have intervened ever since life began, by gradually modifying the atmosphere, which was originally rich in carbon dioxide but wanting in oxygen, and which, astrophysicists tell us, resembled the present atmosphere of Venus. It is probable that bacteria, by the breakdown of carbon dioxide and the liberation of oxygen, provided the essential conditions for the development throughout the earth of life as we recognize it today.

Domesticated by man, bacteria now play an important role by their production, from known substrates, of the numerous chemical substances valuable in agriculture, industry and the different fields of medicine.

Scientific applications

It is the scientific application of their metabolic processes, formerly carried out by craftsmen in a more or less empirical way, that has brought about this impressive development of contemporary industrial bacteriology. Since it is obviously impossible to review all these applications, we must restrict ourselves to mentioning a few of the commoner ones.

The lactic acid bacteria are utilized in the industrial production of lactic acid and in the food industry (yogurt): their specific requirements in amino acids and in vitamins make of these bacteria the micro-organisms best suited to the microbiological proportioning of the growth factors.

The propionic bacteria are responsible for the taste and smell of Emmenthal cheese in which the 'holes' are due to the production of carbon dioxide. These same bacteria are also used in the manufacture of perfumes.

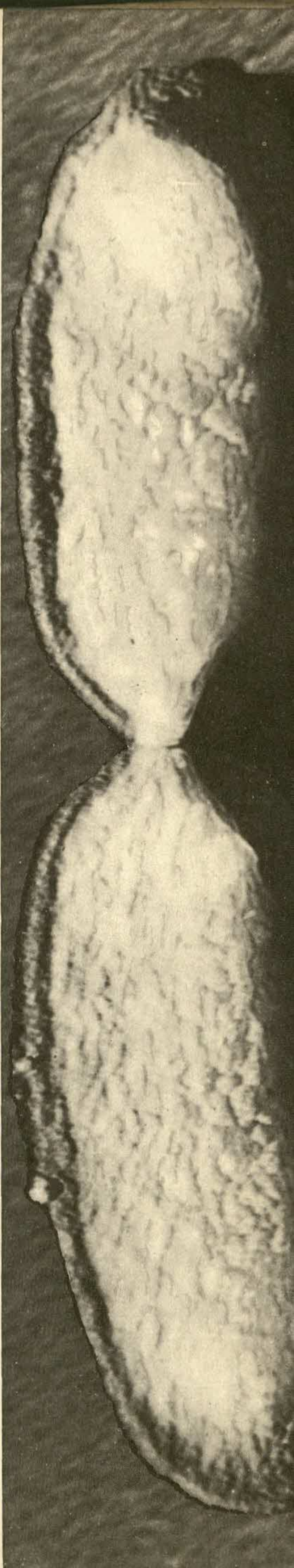
The butyric acid vibrios, whose study led Pasteur to the discovery of 'life without air', are anaerobes capable of producing acetone and butyl alcohol simultaneously from starchy substances.

Certain bacteria, such as *B. polymyxa* and *S. marcescens*, bring about the synthesis from glucose and starchy substances of butanediol which is used in the manufacture of synthetic rubber.

Dextrans, used in human medicine in place of blood plasma, are produced industrially by means of *Leuconostoc mesenteroïdes.*

The production of enzymes of industrial or therapeutic interest (amylases, proteinases, streptokinase) is also effected through bacteria.

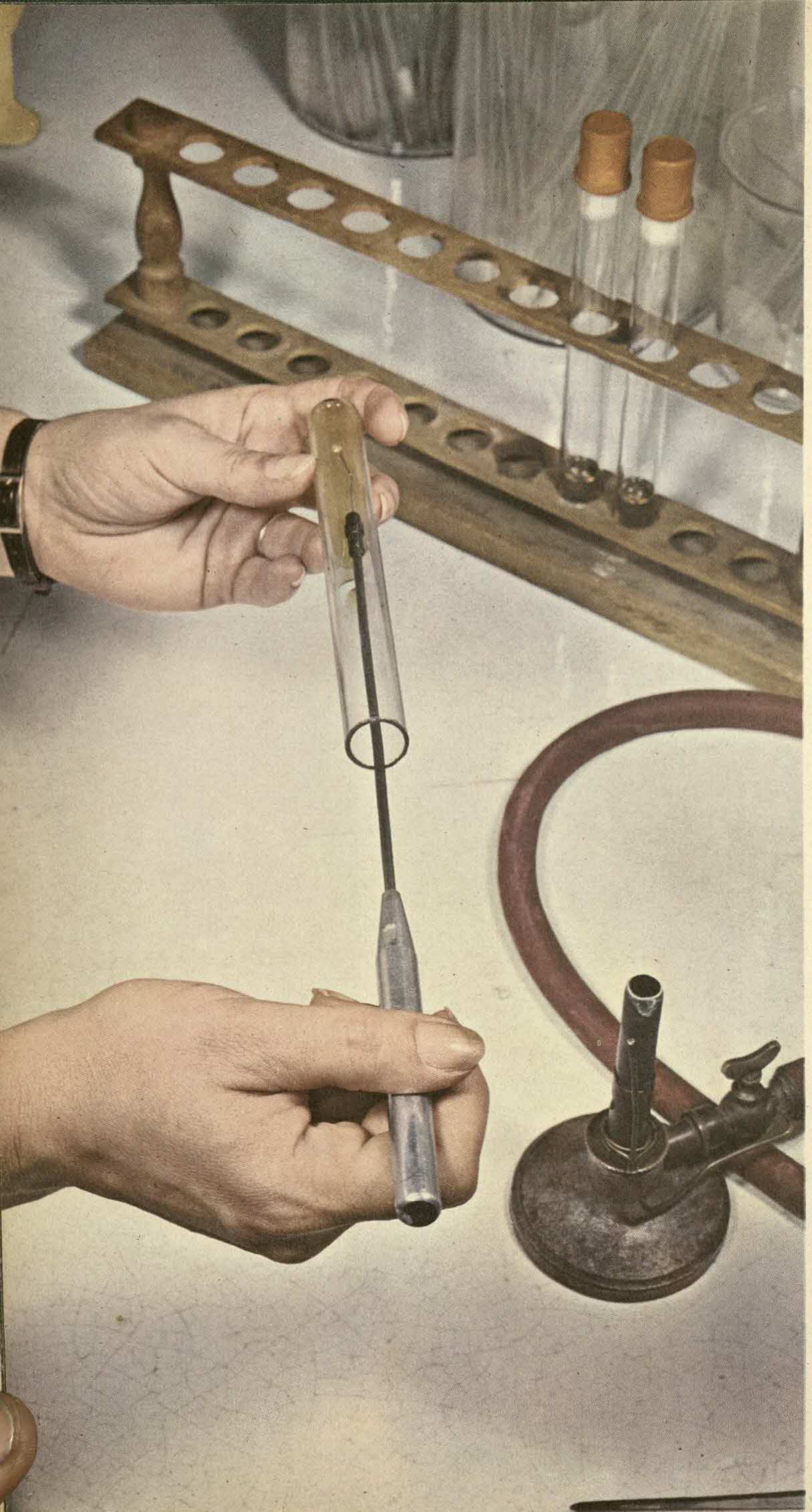
The above examples demonstrate the remarkable powers of synthesis of micro-organisms; they frequently effect, in a single stage, complex transformations which, if chemical methods alone were used, would necessitate a whole series of stages. This is particularly apparent in the production of the steroids hydrocortisone and delta-hydrocortisone that are manufactured by the pharmaceutical industry partly using the intervention of numerous lower fungi and certain bacteria, such as *B. cereus.*



A pathogenic bacillus, Escherichia coli, *at an advanced stage of division. The two daughter cells are almost completely separated. A slender bridge of cytoplasm, ready to break, still joins the two new cells. (Mag.* × *38,000. Istituto Superiore di Sanità, Rome).*

Modern

1



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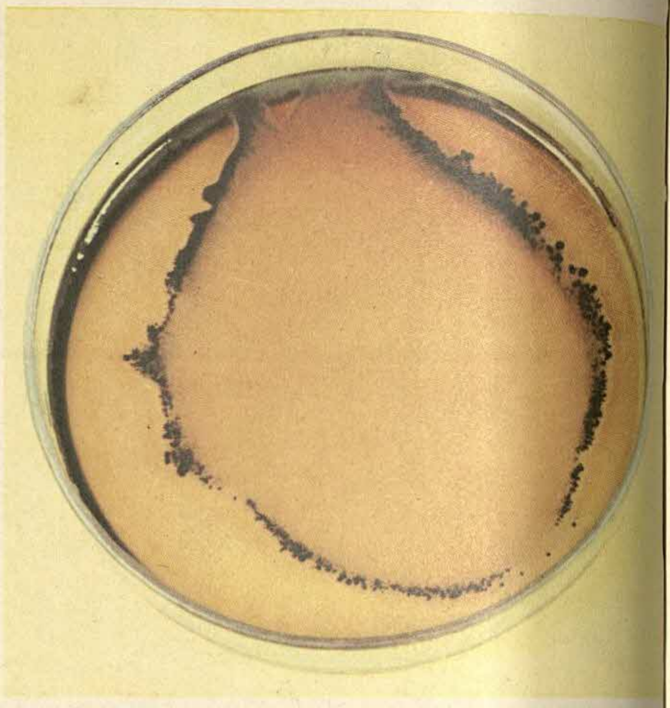

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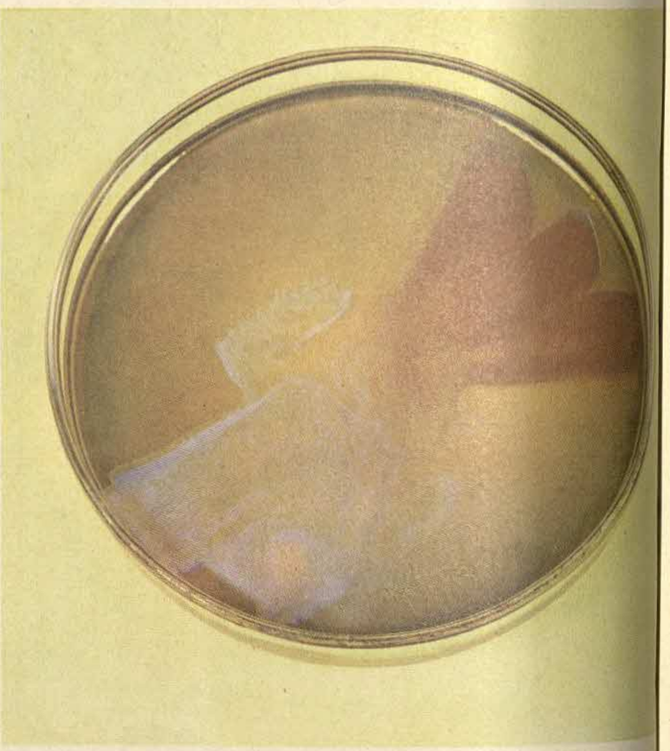
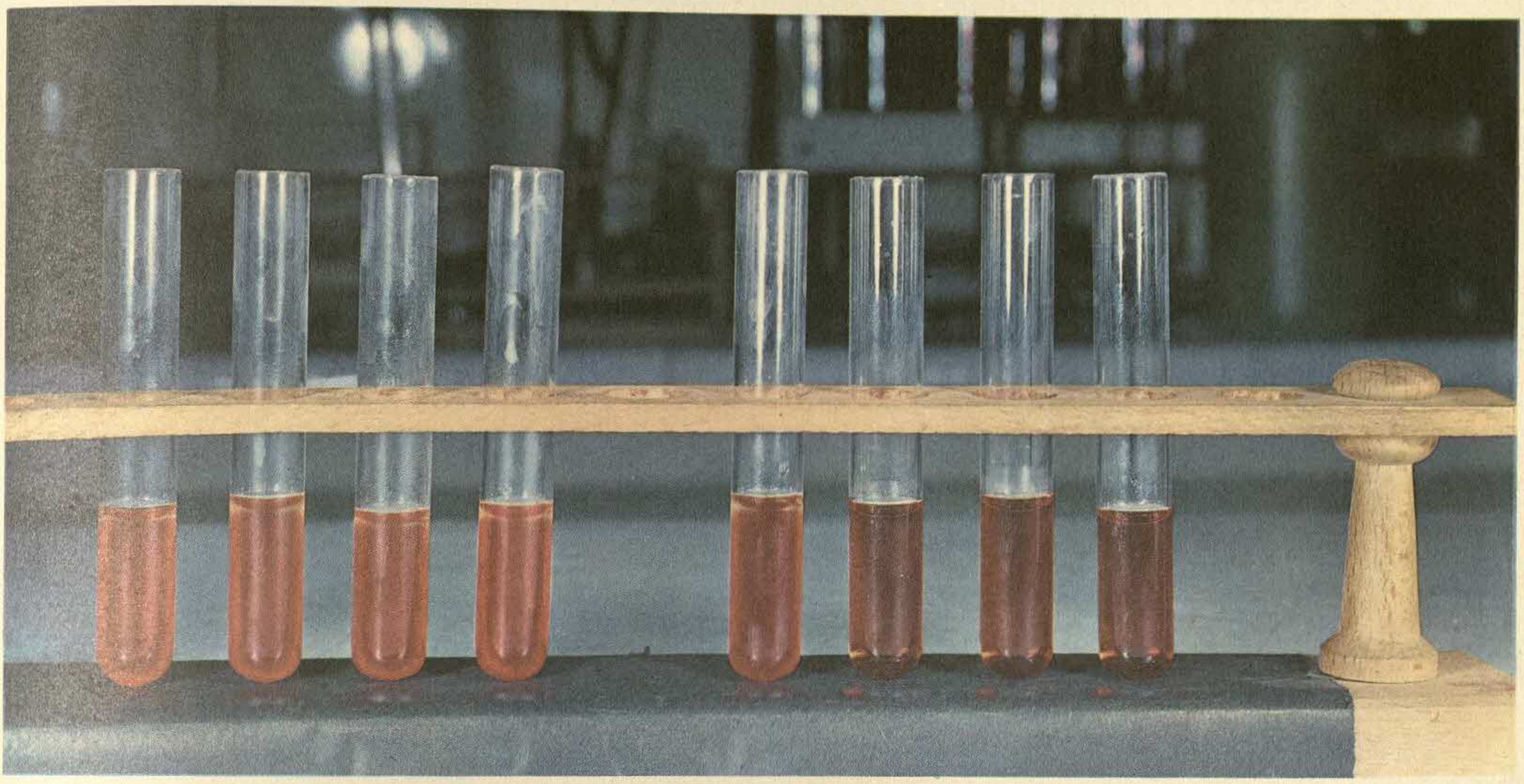


1. This series of photographs taken by Lod at the Institut Pasteur at Garches demonstrates some current work in a microbiological laboratory. Here is an example of a day-to-day operation: the transfer of a culture of bacteria to a new medium. 2. The germs grow and can be seen in the Petri dish. Here the culture is of a pigmented micro-organism, and each little black dot at the periphery represents a bacterial culture. 3. Here, also in a Petri dish, are other cultures of pigmented micro-organisms.

techniques employed by bacteriologists

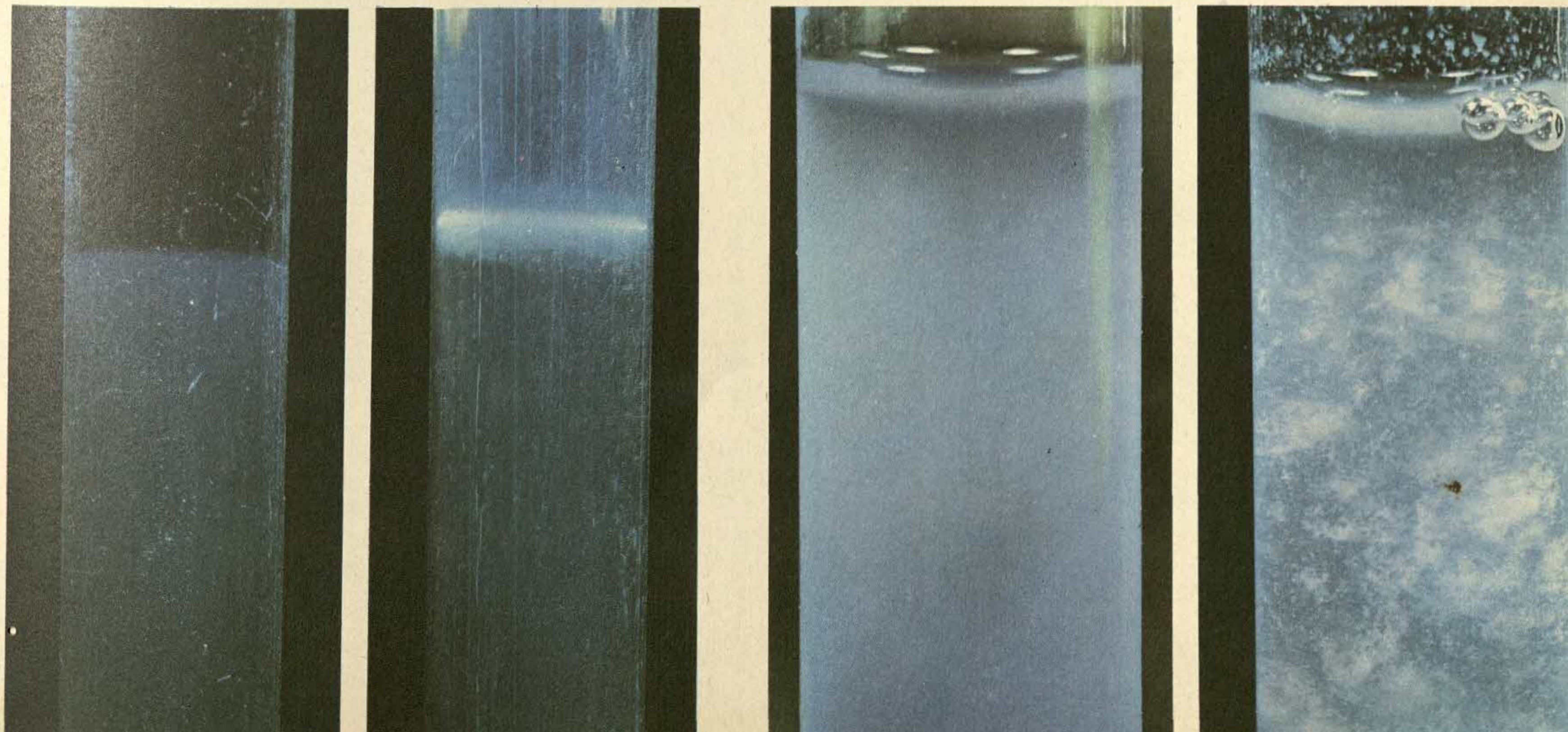
4

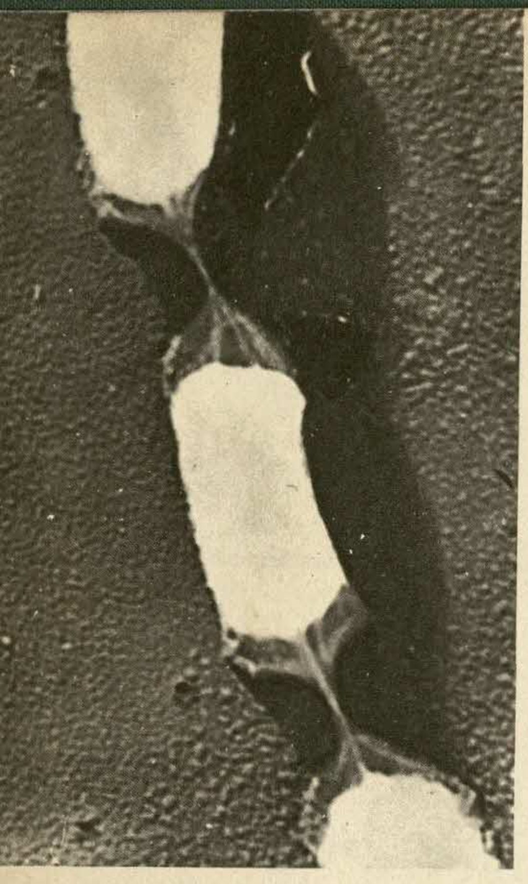


4. Haemolytic reaction of Bordet-Wassermann. Red blood cells, in suspension in the first four tubes, have been destroyed in the last three.

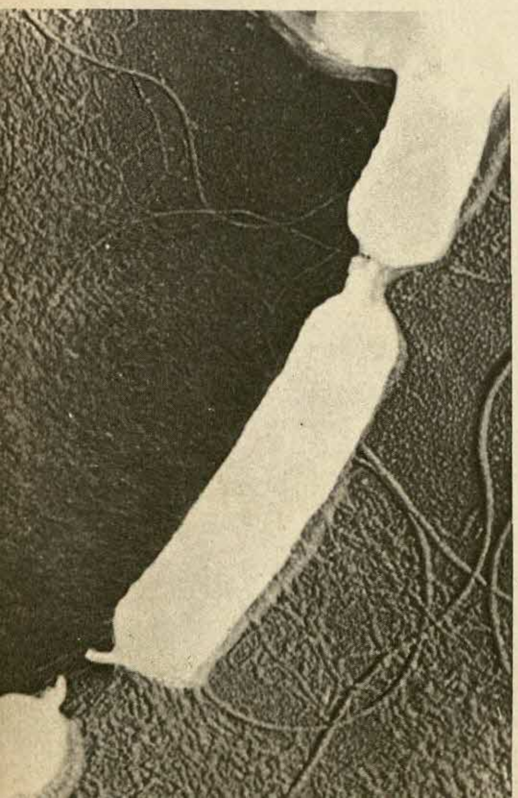
5. An agglutination reaction. Serum is removed from an animal that has been vaccinated against a specific microbe. (1) To this serum are added a few drops of a culture containing the same microbe. It is at once seen that the germs agglutinate rapidly to form visible masses. (2) This is one of the many aspects of antigen-antibody combination which can be carried out in vitro. *The production of antibodies is the object of much research; it is one of the most important factors in immunization.*

5





Division is now almost complete. Thin cellular threads still join the daughter cells. (Mag. × 34,000. Istituto Superiore di Sanità, Rome).



Three bacilli belonging to the species Clostridium botulinum *arranged in file. Two are still joined by a cytoplasmic bridge; the third has just detached itself. (Mag. × 21,000. Istituto Superiore di Sanità, Rome).*

Fleming's discovery of penicillin in 1929 produced by *Penicillium notatum*, and the discovery of tyrothricin by Dubos in 1939, produced by *B. brevis* isolated from soil, formed the starting-point of a vast industry of antibiotic production, which is important not only to medicine but also to agriculture and the food industries.

But the numerous advantages that man has obtained from the rational utilization of bacteria must not allow us to forget the other less comforting side of the picture presented by the world of microbes; for the memorable researches of Pasteur, Bassi, and Koch established that bacteria are responsible for a large number of infectious diseases in man and in animals.

Ever since 1863, the date when Davaine discovered in the blood of animals which had died from anthrax 'a small thread-like body having twice the length of a human red blood cell' and demonstrated that this organism was the cause of the disease, numerous investigations have shown the power of infection of pathogenic germs, and their method of action, and have also demonstrated the defence processes which oppose their invasion.

The discovery by Pasteur of the principle of virus attenuation and of immunization by means of attenuated cultures (1879), of phagocytosis by Metchnikoff (1884), of toxins and antitoxins by Roux, Behring, and Kitasato, of the diphtheria antitoxin by Ramon (1925), of chemotherapy by Ehrlich and of penicillin by Fleming mark the major advances. However, space does not permit further discussion of the vast amount of knowledge gained during the second part of the nineteenth century and the beginning of the twentieth relating to microbes and their pathogenic powers.

Rapid reproduction, intense metabolism

Bacteria have two essential properties which distinguish them among living beings, namely the rapidity of their multiplication and the intensity of their metabolism when placed in favourable conditions of temperature and nutrition. Most bacteria under these conditions reproduce with extraordinary speed. Not more than 20-30 minutes may elapse between two successive divisions, and in six hours a single bacterium can give rise to more than 700,000 descendants. If nothing interfered with this phenomenal capacity for reproduction the entire surface of the earth would be submerged by the descendants of a single individual in a matter of a few days.

The intensity of their nutritive exchanges is no less surprising. Take, for example, the energy produced by the process of oxidation in an aerobic bacterium. If we measure it by the quantity of oxygen absorbed, we find that bacteria are capable of burning in 24 hours a quantity of organic matter comparable to their own weight. By contrast, an adult man, to meet his energy needs, consumes in organic matter only a hundredth part of his weight over the same period.

Because of these properties bacteria are very useful for the study of problems of nutrition, genetics, and research concerned with cellular exchanges.

By reason of their great rapidity of reproduction, bacterial variations arise frequently: hereditary variations due to a change in the genetic constitution, or adaptations to the occasion of new conditions of existence. The bacterial cell possesses, in fact, a considerable number of genes—probably more than 1,000—which correspond to the molecules of deoxyribonucleic acid (DNA) contained in the nucleus. Each gene assures its own duplication and controls the formation of enzymes and other substances responsible for the hereditary characteristics. With a frequency of the order of 1 in 10^8 microbes, spontaneous mutations are produced which have a bearing on the disappearance, the impairing, or the change in position of a gene; and in a single colony of about 10^9 bacteria the various mutations must be reckoned in terms of thousands. These mutations are concerned with certain morphological properties (capsules, cilia, pigmentation), metabolic properties (loss or acquisition of enzymes, resistance to antibiotics), or pathogenicity (loss or attenuation of virulence). They can be induced by physical agents such as x-rays or ultra-violet light, or by chemical agents; but they are not controlled and they appear, as it were, haphazardly.

In contrast, it is now possible to modify at will certain hereditary properties of bacteria by causing deoxyribonucleic acid, derived from a strain previously chosen, to act on them. This acid, after penetrating into the recipient cell, acts there like a gene which is incorporated in the nuclear mechanism, and subsequently reproduces itself in proportion as the cells multiply.

The sexuality of bacteria

The study of genetics has revealed male- and female-type bacteria. There exists, in fact, in certain bacteria, a particular genetic element known as the fertility factor (F) which is independent of other genetic determinants. This is found only in bacteria called F^+ or donating bacteria (called Hfr if they are very fertile, that is to say, of high frequency of recombination). These donating bacteria are capable of functioning as male gametes in relation to the receiving bacteria F^-, which are lacking in this factor.

The mechanism of genetic transfer takes the form of a slow penetration of the donating chromosome into the receiving bacterium, a penetration which may be limited to a fraction of the chromosome.

Bacterial conjugation has been observed under the electron microscope (Wollman and Jacob, 1957): during this process a connection is made at one point on the receiving bacterium's surface through which the genetic material of the donating bacterium is transferred. This method of conjugation is particularly suitable for the study of genetic problems.

It is evident that, in this field, bacteriology has greatly enriched our knowledge. We are now capable of creating at will certain types of bacteria whose new properties are hereditary and indefinitely reproducible.

The bacteriophages

A new era was opened in microbiology with the discovery of a transmissible lysing agent of bacteria. This was due to Twort and d'Hérelle, true pioneers in this field, whose fundamental researches were carried out between 1916 and 1921. D'Hérelle found that by filtering a culture taken from the faeces of a patient suffering from bacillary dysentery, a liquid was obtained which, when added in small quantities to a young culture of dysentery bacilli, led within a few hours to the destruction of the developing bacilli. Furthermore, a filtrate of this lysed culture produced the same phenomenon on a fresh culture. This lytic property is indefinitely transmissible from one culture to another. D'Hérelle put forward the hypothesis that it was due to a micro-organism which parasitized and destroyed the bacteria and then spread and multiplied at the expense of the new bacterial cells. He gave it the name bacteriophage.

This organism, invisible with the means of observation available to d'Hérelle, has since been the object of some very remarkable discoveries made with the electron microscope. Today, it is known that it bears a rough resemblance to a spermatozoon, with a spherical, elliptical or hexagonal head about 50-100 millimicrons in diameter, a tail containing a central axis and a sheath the total length of which may reach 100 millimicrons.

Chemical analysis has shown that the head is formed essentially of deoxyribonucleic acid (DNA) covered by a layer of specific protein which forms the substance of the tail. This protein is contractile within the proximal part of the tail.

Bacteriophages show a great resistance to chemical and physical agents. They can withstand a temperature of 75°C for as long as thirty minutes, and can resist desiccation.

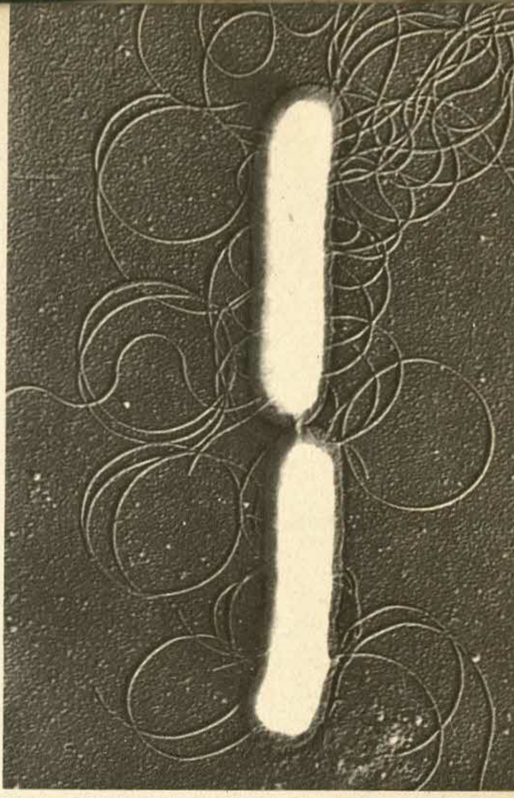
As they are strictly specific, their lytic action makes it possible to recognize the bacteria on which they are active, and this property has been widely used for microbiological diagnosis and epidemiological studies.

When the bacteriophage becomes adsorbed on the membrane of the bacterium by the extremity of its tail, an enzyme analogous to lysozyme intervenes,[1] which destroys a part of the bacterial membrane. A contraction of the protein covering of the tail then pushes deoxyribonucleic acid into the bacterial cytoplasm, injecting it into the organism as effectively as through an ultra-microscopic syringe. Then follows a period of latency, during which the synthesis of protein and nucleic acid in the invaded bacterium is arrested, while that of the phage is effected. When the synthesis of phages thus produced has reached a certain maturity, the bacterial cell is lysed and breaks, occasionally liberating several hundreds of phages which, in their turn, will proceed to invade new bacterial cells.

The duration of this cycle is about twenty to sixty minutes.

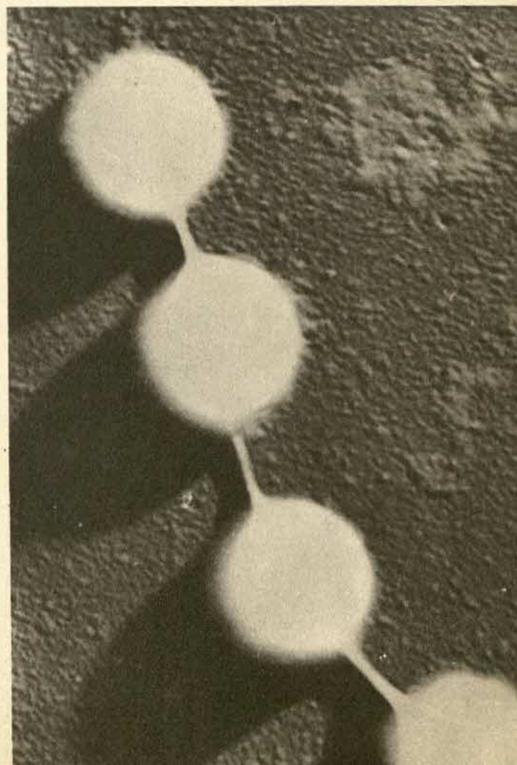
A remarkable fact is that the phage, while penetrating into a bacterium, can transmit genetic material coming from one which it has previously invaded. This phenomenon of genetic transduction may give rise to a controlled mutation provided, of course, that the receiving cell is left unlysed. Certain specific properties (antigenic, metabolic, resistance to antibiotics) can be induced by this mechanism.

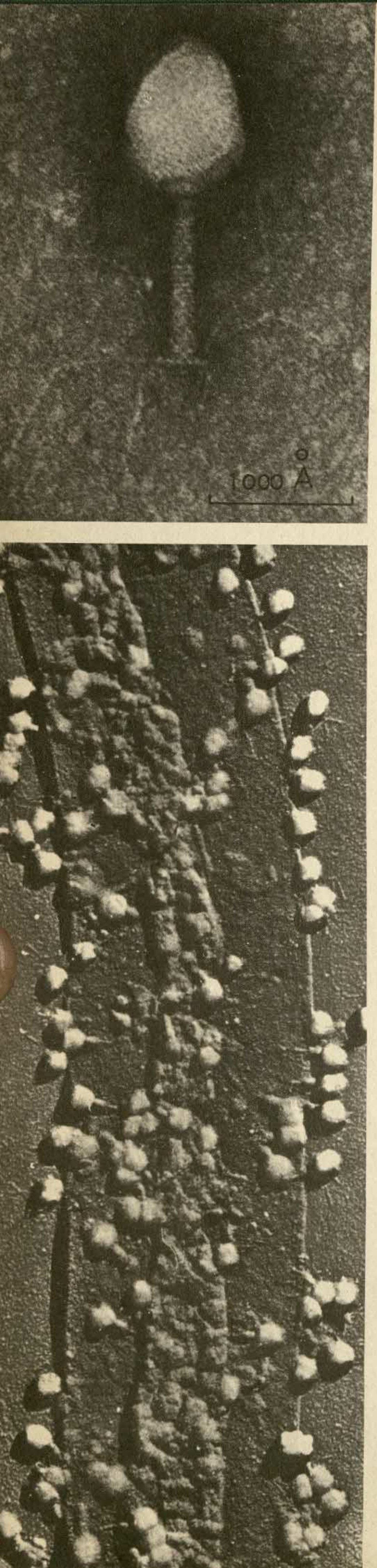
These findings bear on a discovery, of considerable interest from the biological point of view, made by Lisbonne and Carrère in 1922. These researchers had noticed that certain bacteria, called lysogenic strains, could be invaded by a phage, called a temperate phage, without any apparent effect on the bacterial cell, which continued to reproduce



Anaerobic bacillus. Note the characteristic circular shape of the cilia. The two cells still have a cytoplasmic bridge joining them. (Mag. × 31,500. Istituto Superiore di Sanità, Rome).

The division of cocci at its final stage. Each cell is still joined to its neighbour by a thread of cytoplasm. (Mag. × 31,500. Istituto Superiore di Sanità, Rome).





Escherichia coli, *surrounded by bacteriophages. The cell walls are emptied, the sheath contracts. Above—a bacteriophage under very high magnification. (Mag. × 260,000 and 42,000. Laboratory of Biophysics, University of Geneva).*

normally. The daughter cells carried this phage genome which reproduced itself at the same time as the bacterial genome; it is capable in certain circumstances—under the influence of ultra-violet rays, for example—of being transformed into a mature phage. The name prophage is given to this constituent, and lysogenization to the phenomenon.

Bacterial cells, thus invaded in a latent fashion by the prophage, are immunized against infection by a phage of the type which they carry.

There is a striking analogy between the phenomenon of lysogenicity and latent infection by animal viruses. However, what is more remarkable is the fact that these apparently normal cells can be the agents of transmission of certain genetic substances from one bacterium to another. This transduction by prophage is of considerable biological interest; for it is in this way that non-toxic strains of diphtheria bacilli can acquire the property of producing toxins when they are submitted to the action of prophages coming from toxic strains, and similarly, some *Salmonella* can acquire new somatic antigens by an analogous process.

Thus the discovery of bacteriophages has opened for biologists entirely new avenues relating to the plasticity of living matter, and has provided them with methods of study from which the later researches on viruses have greatly benefited.

What is a virus?

Among the infectious diseases of man and animals there are many in which a bacterial cause has not been found. These diseases have been known since the earliest times, some being very contagious and capable of causing serious epidemics, such as smallpox, whose highly infective nature has long been known.

It is also known that rabies is contracted from the bite of a dog, a bite that the Hebrews compared to that of a poisonous snake. When Pasteur tried—in vain—to reveal in the blood or organs of animals suffering from rabies a microbe that could be its cause, he suggested that it must be due to a microbe too small to be detected by the microscope.

These entities, as yet undiscovered in the time of Pasteur, are, in fact, very widespread in nature and have since been given the name of viruses. But it is necessary to recall that this very general term meant, at that time, all the pathogenic agents of infectious diseases, whether bacterial or not. The Latin word *virus* means many things such as foam, poison or venom. *Volucri ferro tinctile virus inest*, wrote Ovid.

In fact, the existence of a sub-microscopic virus was demonstrated in 1892 by Ivanovsky, who proved that the disease of tobacco leaves, known as mosaic disease, was due to a pathogenic agent so tiny that it could pass through the pores of a filter impervious to any known bacteria. Some years later (1898) Loeffler and Frosch, on the one hand, and Sanarelli, on the other, showed the same filter-passing property of a pathogenic agent in the cases of foot-and-mouth disease and myxomatosis of rabbits respectively. In 1901, research on yellow fever by the American Reed and his colleagues led to the same conclusion.

Since then, very many filterable microorganisms have been obtained from the most diverse sources, and it is to these that the word virus now refers. Their study, named virology, younger sister of bacteriology, has in the last few years taken the lead among the biological sciences.

Viruses are not bacteria of small size. Their structure and properties are quite different.

Their dimensions are expressed in millimicrons (millionths of a millimetre). The largest are in the region of 200-300 mμ and can be seen with an ordinary microscope (vaccinia virus). The smallest are in the region of 10 mμ or, in other words, about the size of large albumin molecules (foot-and-mouth disease virus) and are visible only under an electron microscope. Viruses are incapable of growing on the usual bacterial culture media, and can proliferate only in living cells. They are specific and can give rise to infections in man and mammals, plants and insects.

Great progress has been made in the study of human and animal viruses by culturing them on egg embryos and, more recently, on artificial tissue cultures. This latter technique has revolutionized virology and has led to the isolation of a multitude of new strains of viruses, some of which appeared for a long time to be without any pathogenic properties (orphan viruses). The utilization of certain strains of cells cultivated in uni-cellular layers, such as the HeLa strain issuing from an epidermoid cancer of the neck of the uterus, has made the use of this technique still more suitable. Today it is easy to grow artificially a large number of viruses on the cells of monkey kidney, or on amnion, or thyroid removed from man.

The development of viruses on the cells during culture leads to their being impaired and often to their being destroyed. This cytopathogenic effect, which varies according to the virus, is visible to the naked eye. It is seen by the appearance of plaques (Dulbecco) comparable to those produced by bacteriophages; it is possible, by this process, to assess the number of viral particles developed in the culture.

Viruses and nucleic acids

Researches on the physico-chemical nature of viruses have produced some remarkable discoveries. All viruses are composed essentially of nucleoproteins formed from a single nucleic acid which is ribonucleic acid (RNA) for plant, influenza and poliomyelitis viruses, and deoxyribonucleic acid (DNA) for most animal viruses. It is this nucleic acid which is the infective part of the virus. In 1935 Stanley showed that the tobacco mosaic virus could be crystallized: and in 1955 Williams and Fraenkel-Conrad, by joining some protein with a chemically isolated nucleic acid, remade a virus which reproduced particles in the form of rods, almost identical to those of the natural virus, which gave rise on tobacco leaves to lesions characteristic of the disease.

The production of a substance endowed with properties usually considered to be those of living matter marks a memorable step in the story of biological knowledge.

Comparable experiments have been carried out with the virus of mouse leukaemia (Latarjet) and with the foot-and-mouth virus.

It is possible that a new step will be taken when, with a better knowledge of the chemical structure of the ribonucleic acid concerned, we are able fully to effect the synthesis.

Many researches are at present being carried out along these lines. But perhaps in the synthesis of such a virus the spatial arrangement may be more important than the chemical constitution. Thus we know that the tobacco mosaic virus is formed in a spiral structure enclosing threads of ribonucleic acid.

Some very recent studies, made with the electron microscope, of myxovirus and adenovirus stained with phosphotungstic acid (Brenner and Horne), have shown that they are composed of a central nucleic acid surrounded by a protein envelope. The shape of this approaches that of a regular icosahedron; in other words, it is seen, under the electron microscope, as being bounded by twenty equilateral triangles.

Recent research in this field has attained a high degree of precision, and it is surprising to find that living beings, capable of indefinite reproduction and of giving rise to infectious diseases in a similar way to the bacteria, can occur in the form of crystals, a form which we usually associate with the structure of essentially inanimate objects.

In spite of their crystalline form, viruses are essentially plastic. They reproduce by auto-duplication. They are also the object of mutations resulting from processes comparable to those which control the biology of the bacteriophages. These mutations can be directed, and it is in this way that virology can usefully interfere in order to modify the pathogenic power of certain viruses and transform them into beneficial vaccines.

It has thus been possible to obtain from the poliomyelitis virus some mutants of attentuated virulence which have been used in the living state, taken by mouth, as vaccines against this disease. Similarly, by culturing on an egg, we can grow attenuated strains of the viruses of rabies and bovine plague, and in this way veterinary medicine has been provided with vaccines of great efficacy.

Like the prophages, viruses are able to integrate themselves into a host cell and live there in a latent state (provirus); they are transmitted to the offspring of the cell and can reappear—apparently spontaneously—under the influence of an inducing shock.

Immunity against viruses

In general, living cells can be infected by only one virus at a time, unless there are two viruses of which one develops in the nucleus and the other in the cytoplasm. This is the case, for example, with the viruses of herpes and vaccinia, of measles and poliomyelitis. But usually inoculation of a virus makes the cell resistant for a certain time to the effects of a second or several other virulent viruses. This fact is easy to demonstrate in tissue culture. Thus, infection by the virus A of influenza prevents a subsequent invasion by the mumps virus. There is every reason to think that such phenomena occur naturally in man, and that they play an important part in immunity against viral infections.

Plant diseases can result from the action of viruses. Here is the agent of cymbidium mosaic with a fibrillary structure. (Mag. × 40,000. French National Institute for Agronomic Research).

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Micrograph of the virus of turnip jaundice, stained with phosphotungstic acid, and enlarged 85,200 times. (French National Institute for Agronomic Research).

Interference of this sort is found even between viruses and their own mutants. It is in this way that the dermotrophic virus of vaccinia prevents the development of its neurotrophic variant, and the non-encephalitogenic strains of herpes protect the rabbit against the effects of the virulent encephalitogenic strains.

Killed viruses possess, like living viruses, this capacity for interference. For example, when influenza virus, after being killed by heat or ultra-violet rays, is incubated *in vitro* with the chorio-allantoic membrane of a chick embryo, a substance is produced which has the property of interfering with the influenza virus. This substance, called 'interferon' (Isaacs and Lindenmann, 1957) can be isolated from tissues. It is a stable protein, capable of inhibiting the development of a very large number of viruses.

The researches in progress are full of promise. It is possible that interferon may be capable of preventing—or perhaps even curing—a great number of human and animal viral infections. This discovery should be all the more valuable since these infections are generally resistant to the usual antibiotics.

Viruses and cancer

A last question on the subject of virus infections: can recent knowledge throw some light on the problem of cancer?

It has been known for some time that viruses exist which are capable of giving rise to cancer in certain animal species. But very little is known of the manner by which they determine the anarchic proliferation of the invaded cells.

Experiments have shown that it is possible to transform in tissue culture a carcinogenic virus (Shope's fibroma) into another virus (that of myxomatosis) by the action of preparations of the latter virus killed by heat, (Kilham). They evoke the mutations well-known in viruses.

An even more interesting fact is that we can successfully transmit cancer to adult mice by the injection of chemically pure DNA extracted from the normal organs of non-cancerous mice which, however, have arisen from a strain in which cancer is frequent. The virus can lie latent and the characteristic lesions appear only under the influence of certain stimuli such as hormones or carcinogenic substances. One can see the analogy with the provirus.

In 1961, Dalldorf, Doolan and Moore succeeded in extracting from human tumours a factor which, when inoculated into new-born hamsters, gives rise to an infection that is characterized by severe malformations and which can very quickly turn into a fatal disease. The agent causing this has all the physico-chemical characteristics of an infective virus of small size.

These researches open the way to useful and fruitful investigations in this field of science.

Structural continuity of the world of microbes

If we envisage the whole world of microbes—grouping under this title all living organisms of microscopic or sub-microscopic dimensions—we are at once struck by the structural continuity which they present.

The largest among them, the bacteria, are formed of proteins, carbohydrates, lipids and enzymes which enable them to assimilate nutritive matter from their environment. But their almost incredible power of multiplying is assured by their equipment of nucleoproteins, notably ribonucleic and deoxyribonucleic acids, whose role is vital in the life and reproduction of their cells and which connect them chemically to the genes of animal and plant cells.

When we pass from the bacteria to viruses—and as Minck and Tulasne have established, there exist intermediate forms, the L forms, which are capable of reverting to the bacteria of origin—we cannot but note the increasing relative importance of nucleoproteins, until we come to the point where certain viruses are no more than pure nucleoproteins. This primitive structure forces them, in order to multiply, to become parasitic upon living cells in order to use their chemical activity for their own profit. The continuity throughout the world of microbes of this nucleoprotein structure, indefinitely reproducible and apparently the fundamental element, is very striking.

Although the researches of Pasteur have taught us that spontaneous generation does not exist and that every living being is born from another living being, it is nevertheless true that if man succeeds in synthesizing this elementary viral particle which now seems to be one of the most rudimentary forms of life, the dogma of spontaneous generation may have to be reconsidered.

PART ONE

The bacterial cell

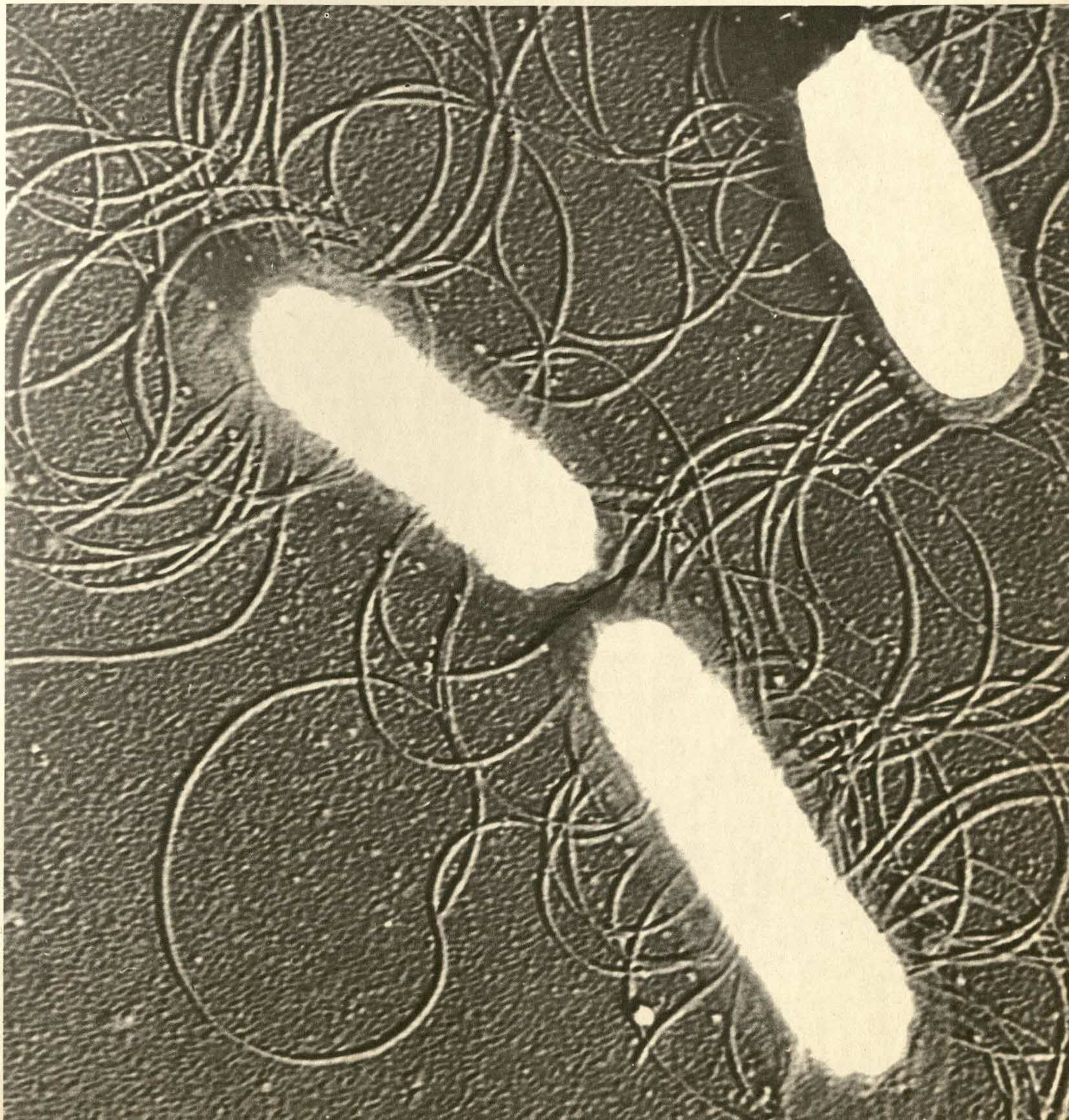


Fig. 1. Bacterial cells magnified 31,500 times. Istituto Superiore di Sanità, Rome.

The physicochemical structure of bacteria

Wayne W. UMBREIT

Bacteria[1] are, by definition, very simple microscopic organisms devoid of chlorophyll and which reproduce by division. They are the smallest known cells, with a cellular organization. They have all the physico-chemical properties of cells, but their internal structure must be distinguished from that of other cells. Bacteria occur in three shapes: spheres (called *cocci*,[2] singular *coccus*), rods (called *bacilli*,[3] singular *bacillus*), and spirals which may be regularly curved and which range in form from a 'comma' to a coiled spring. Dimensions are variable. A sphere has normally a diameter of 0·5 microns; a rod has generally a diameter of 0·5 microns but its length (or height) may be from one to several microns.

However, the size and shape, while they vary over certain limits, are not the critical factors which control the bacterial structure. Chemically, the spheres are more like certain of the rods which differ markedly from other bacilli. In fact, as well as by size and shape, bacteria are divisible, upon the basis of their physico-chemical properties, into two large groups. These groups differ in fundamental structure and in their metabolic character, and, undoubtedly, as a reflection of these factors, they differ in their sensitivity to antibiotics, to disinfectants, to the environment in which they can live, etc. There is, in addition, a 'simple' staining method which, because it reflects this physico-chemical difference, differentiates bacteria into two general types. This stain is called the Gram stain (named after the man who discovered it) and it consists of treating a heat-fixed smear of the bacteria with crystal violet, then with an iodine solution, then with 95 per cent alcohol. and finally with some stain of a colour contrasting with the violet. Two kinds of result are found: some bacteria are violet; these are called 'Gram-positive'; other bacteria are not violet but are the colour of the 'counterstain'; these are called 'Gram-negative'.

The 'Gram-positive' and 'Gram-negative' types of bacteria have several things in common, but they do differ in certain fundamental aspects. All bacteria, whether Gram-positive or -negative, have a rigid cell wall. It is the rigidity of the cell wall which characterizes bacteria; indeed, they possess a structure different from other living creatures. The animal cell wall is a non-rigid structure, in which are located the permeability barriers of the cell. The plant cell wall, while rigid, is composed of cellulose, and beneath this structure there is a membrane in which the permeability of the cell is controlled. The bacterial cell wall is also rigid, but has no trace of cellulose. In fact, it is in the nature of the substances in the bacterial cell wall that a part of the fundamental distinction between Gram-positive and Gram-negative cells lies. Beneath the rigid cell wall of the bacteria there is also a cell membrane in which are the permeability properties of the cell, but which also is enzymatically active and which contains many, if not all, of the respiratory enzymes of the cell.

The capsule

To understand the physico-chemical structure of bacteria, it is convenient to begin by examining the outside. The first sheath met with is generally called the 'capsule' and is microscopically demonstrable as gelatinous material associated with and surrounding the cell. It is not apparent in any of the figures of this article. It may vary from a very thin, barely detectable layer to a mass of material greater than the cell itself. In many cases it has a definite external boundary; in other cases, the material seems to be diffuse. The capsule is not an essential and integral portion of the cell, as are the cell wall and cell membrane, and it may be dispensed with on occasion. Many organisms may possess no capsule; in others, capsulated bacteria may have mutants which continue to live in the capsule-free state. Capsules are possessed by both Gram-positive and Gram-negative bacteria. They are composed of polysaccharides[4] or polypeptides,[5] poor in amino acids. (It can happen that there is only one amino acid.)

The cell wall

Underlying the capsule is the cell wall itself. Its characteristic property is that it is rigid, but its chemical composition differs markedly between the Gram-positive and Gram-negative bacteria. We shall first describe

(1) Bacteria *are single-celled, microscopic organisms, devoid of chlorophyll, and reproducing by fission.*

(2) Cocci *are spherical bacteria.*

(3) Bacilli *are rod-bacteria.*

(4) *A* polysaccharide *is a substance composed of many molecules of sugar (or sugar acids) joined together.*

(5) *A* polypeptide *is a substance composed of many molecules of amino acids joined together.*

(6) Muramic acid *is one with nine carbon atoms derived from an hexosamine found in the cell walls and spores of bacteria.*

(7) *An* hexosamine *is a sugar with an amino radical.*

(8) *An* aromatic amino acid *is one with a cyclic nucleus (tyrosine, phenylalanine, or tryptophane).*

(9) Proline, *an amino acid.*

(10) Histidine, *an amino acid.*

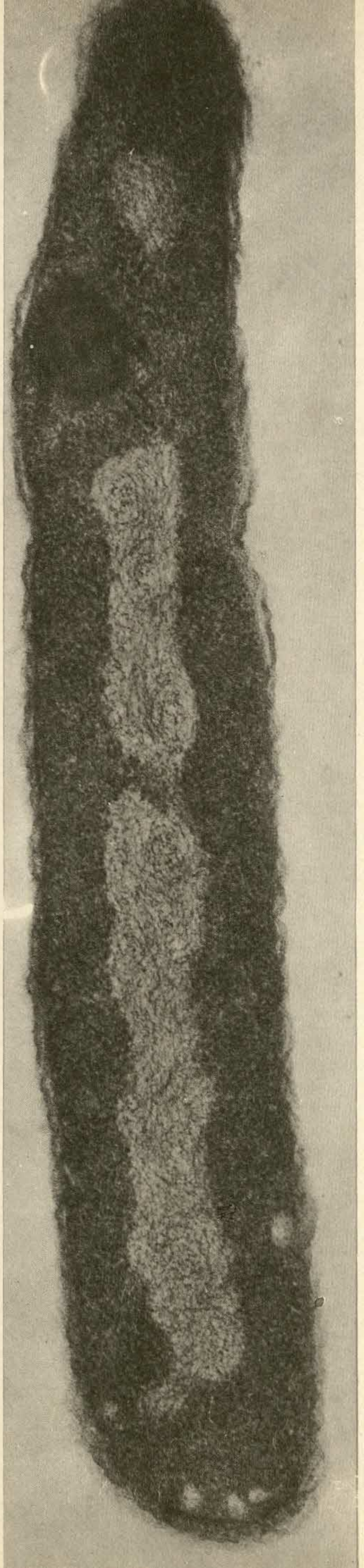
(11) *A* protoplast *is a cell from a Gram-positive organism, without its cell wall. A* spheroplast *is a cell from a Gram-negative organism, without the rigid material of its cell wall.*

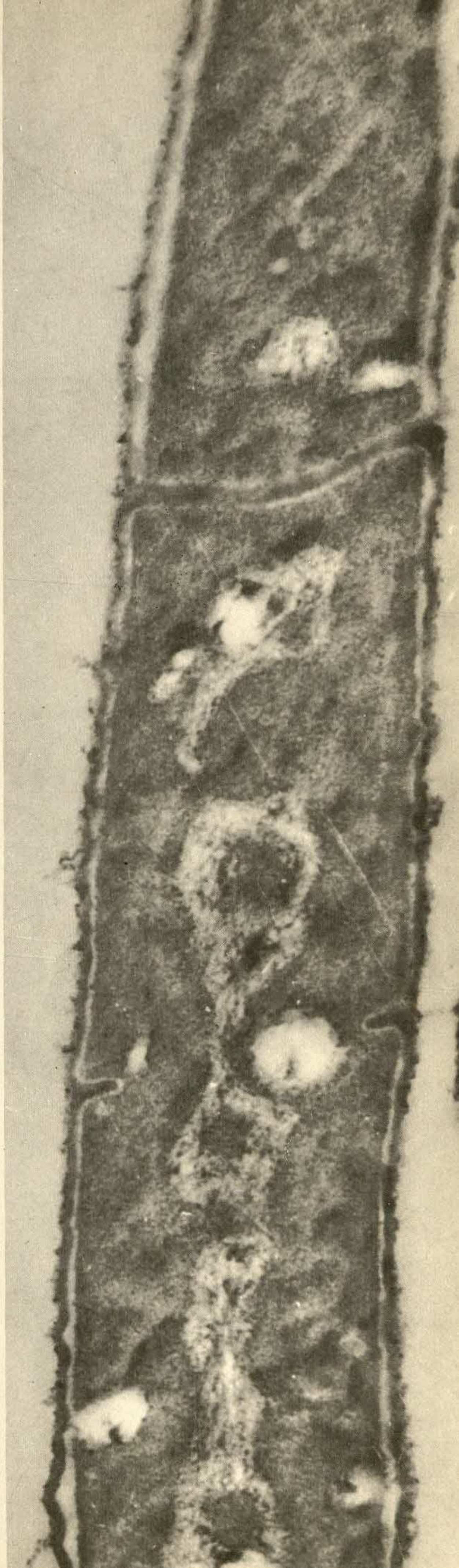
Fig. 2. (opposite page) Fine bacterial sections. Right: section of Bacillus cereus. *The cellular membrane, a rigid envelope which characterizes the bacteria, is clearly visible, as well as a complete transverse division. Below are the two starting points of a second division. (Mag. × 36,000). Left:* Escherichia coli *(mag. × 40,000.). (Photo Laboratoire de Biophysique, Geneva—Chapman and J. Hillier, 1953).*

the properties and composition of the cell walls of Gram-positive bacteria. In fig. 1, we have reproduced a photograph of a crushed cell preparation of a Gram-positive organism. In this case, the crushing has broken some of the cells, and the protoplasm has been lost from several broken cells, but the cell wall remains, a rigid tube. Fig. 2 is a photograph of an ultra-thin section of *Bacillus cereus*, the same organism as in fig. 1, magnified under the electron microscope 36,000 times. One may note that in places the protoplasm has pulled away from the cell wall, leaving it clearly separated. It has been possible to fix the membranous coverings by centrifugation of cells already dissociated by mechanical means, and so to study their physico-chemical characteristics. The cell walls of several Gram-positive bacteria are between 15-20 millimicrons in thickness (10-15 in Gram-negative bacteria). Thin sections of cell walls seen under the electron microscope indicate a somewhat greater thickness. Physically, the cell walls are rigid tubes. At very high magnifications, some show geometric patterns, eg hexagonal ones; others are entirely amorphous.

In Gram-positive cells, the cell wall has a high content of 'hexosamine'; this proved on further study to be a polymer of glucosamine and 3-O-acetylcarboxylethyl hexosamine, called muramic acid[6] (fig. 3). The 'hexosamine'[7] content of cell walls of Gram-positive cells is of the order of 16 to 20 per cent of dry weight, in contrast to Gram-negative cell walls where it is 2-5 per cent. In Gram-positive cell walls there are a limited number of amino acids (from 4-10) and there are no aromatic[8] or sulphur-containing amino acids, no proline,[9] no histidine[10] and no arginine among them. Lipid content is low (of the order of 1-2 per cent dry weight). Some are rather high in phosphates, and a study of this phenomenon revealed the presence of two substances: a ribitol phosphate polymer and a glycerol phosphate polymer, each called 'teichoic acid'. It is not known how these components connect to form the cell wall structure, but it appears that the rigidity of the wall is due to the muramic acid complex, to which are attached some of the amino acids in peptide links (fig. 4); but the detailed chemistry is not known.

The Gram-negative cell wall differs from the Gram-positive cell in a variety of ways. For example, it has a lipid content of 10-20 per cent (compared to 1-2 per cent in Gram-positive cell walls), with 2-5 per cent hexosamine. The amino acid content is considerably higher, with those amino acids





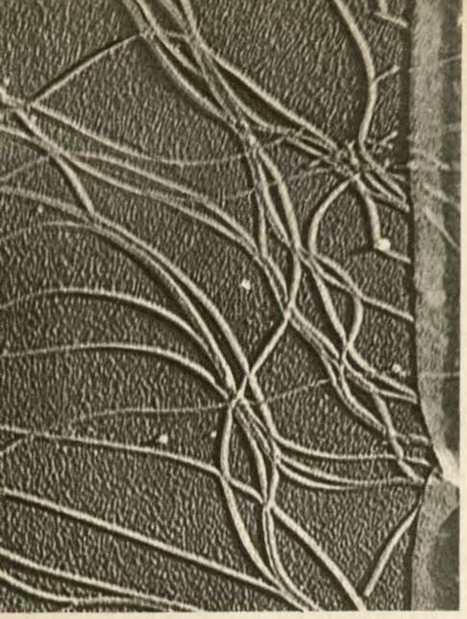


Fig. 3. New techniques of preparing bacteria and observing them through the electronic microscope reveal details more accurately than ever before. (Bacillus megatherium).

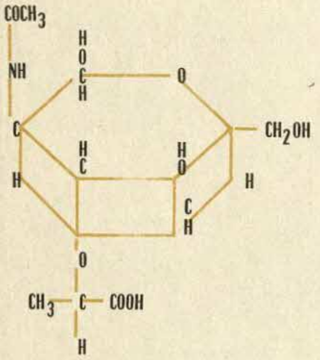


Fig. 4. Muramic acid.

characteristic of most proteins. From related studies, it appears that the Gram-negative cell wall has a structure like that of the Gram-positive cell (and it is this structure which is responsible for the rigidity of even Gram-negative cell walls), but that the 'positive-like' structure is only about 10 per cent of the cell wall and the remainder is composed of proteins and lipids which are 'absent' in Gram-positive cells.

It has been found possible to remove or to prevent the formation of bacterial cell walls. Under these circumstances, the cell bursts ('lysis'). But if such cells are protected by a properly balanced salt or sugar solution, a cell-wall-free cell[11] may be obtained. The basis for these methods of removing the cell wall is as follows. First, the muramic acid. complex structure is attacked by lysozyme (an enzyme occurring in egg white), and when this structure is removed, the rigidity of the cell wall is lost (in both Gram-positive and -negative cells) and all cells become spheres. One may also prevent the formation of the rigid muramic acid complex by means of penicillin; indeed, this prevention of bacterial cell-wall formation appears to be the mode of action of penicillin in killing bacteria. In this case also, all cells become spheres. Such cell-wall-free preparations of spheres protected by proper osmotic solutions, produced from most Gram-positive organisms, are called 'protoplasts' and appear to be substantially free from the chemical materials found in cell walls. The protoplast still has the permeable property of the intact cell; it can metabolize, it can synthesize materials and it can even divide. Prepared from Gram-negative cells, such spheres, protected by the proper osmotic solutions, are called 'spheroplasts' and while they are essentially free from the muramic acid component, they may still contain the protein and lipid materials which comprise the non-rigid portion of Gram-negative cell walls. The muramic acid structure, responsible for the rigidity of the bacterial cell wall, has not yet been found elsewhere in nature and appears to be unique for bacteria (or closely related micro-organisms).

Cellular membrane and protoplasm

Beneath the rigid and unique cell wall there is a cell membrane (fig. 2). In both Gram-positive and Gram-negative bacteria this seems to be essentially similar and in it are two necessary properties of the bacterial cell. The first is the property of differential permeability[12] which in bacteria may be any of four different types. There may be simple diffusion, and diffusion requiring energy, a stereospecific entry, and a stereospecific entry requiring energy. By *stereospecific* one means, in this connection, that related molecular structures may be selectively taken up by the cell, and whether the cell will permit entry or actively concentrate a given substance depends upon its configuration in space rather than upon its molecular size, solubility, etc.

The second property of the cell membrane appears to be relatively unique. The cell membrane of bacteria appears to contain *all* of the respiratory enzymes[13] of the cell. It appears that bacteria may not contain mitochondria, which are structures within the protoplasm of animal and plant cells containing the respiratory enzymes. Instead, bacteria have all of these enzymes in the cell membrane. There has been evidence of 'mitochondria' in bacteria, but it appears today that such 'mitochondria' were actually cell wall fragments rather than pre-existing structures in the cell cytoplasm.

Beneath the cell membrane is the 'protoplasm' and in it one might expect to find a nucleus. The bacteria are so small, however, that the nucleus is not visible under the optical microscope and is usually too similar to the rest of the cell to be differentiated under the electron microscope (fig. 2). Hence, all evidence for a nucleus must be somewhat indirect, but it is nevertheless clear that bacterial cells contain a nucleus. Two other structures are found in some bacterial species. Flagella are long protein-containing appendages which are involved in the movement of motile strains. A few bacteria form spores, one spore within a cell. Such spores are more resistant to heat and to drying than are the non-spore-containing cells. It is curious that the cell walls of spores contain the muramic acid complex characteristic of the rigid bacterial cell wall.

Subsequent sections of this work will give more information on Gram-staining, protoplasts, etc. But at this point, two distinct characters of the bacteria should be emphasized. They are unique for bacteria and are known nowhere else in nature. First, the bacteria have a rigid cell wall. This rigidity is due to the presence of a glucosamine-muramic acid complex which exists only in the cell wall of micro-organisms. Second, the bacteria have a non-rigid cell membrane, osmotically active, which contains the respiratory enzymes.

(12) Differential permeability *means that some substances penetrate, while others do not.*

(13) Respiratory enzymes *are elements which permit the cell to absorb the oxygen in the air to obtain energy for its needs.*

(14) Mitochondria *are small structures in the protoplasm of higher animal and plant cells, and which carry the respiratory enzymes.*

The importance of endotoxins

Albert DELAUNAY

Under the name of endotoxins are designated certain constituents lying at the surface of the bacterial cell and having antigenic and toxic properties. That is to say, they make the organism form antibodies. Their importance for the microbiologist, pathologist and physiologist is considerable.

The endotoxins, like the exotoxins,[1] are bacterial poisons, but these two groups of substances differ from one another fundamentally.

Exotoxins are produced by only a very small number of germs such as the diphtheria bacillus or the tetanus bacillus; on the other hand, innumerable bacteria possess an endotoxin. No sooner are they formed than the exotoxins are released into the infected organism (diphtheria and tetanus are basically intoxications) or into the media of artificial culture. Endotoxins, on the contrary, *in vivo* as *in vitro*, are freed only by dead germs in the process of autolysis. The exotoxins are powerful antigens or, to put it in another way, they cause the infected organism to elaborate an antidote known as an antibody. The antigenic power of endotoxins is, comparatively speaking, mediocre.

The exotoxins, or at any rate the most common ones, are highly poisonous; but this is not the case with the endotoxins, which prove lethal only in relatively high concentrations. Each exotoxin produces in sensitive animals a state of intoxication peculiar to itself; thus the diphtheria toxin, but not the tetanus toxin, is damaging to skin. Nothing comparable to this is found with the endotoxins, which produce the same morbid symptoms no matter which germ is involved. One can, by treating an exotoxin with formol and heat, dissociate its antigenic from its toxic properties; the second is suppressed, the first persists. A new body, or anatoxin (Ramon), has thus been formed which is an extremely efficacious type of vaccine, but though attempts have been made to obtain anaendotoxins under the same conditions, the results have been disappointing.

Composition of endotoxins

Certain bacteria, coloured by gentian violet, retain this colour when they are subsequently treated with iodine and alcohol. These are described as Gram-positive, whilst the others, which are decolorized, are called Gram-negative. Most of the germs which secrete an exotoxin are Gram-positive. However, the Gram-negative germs are alone in possessing an endotoxin: belonging to this group are, for example, the paratyphoid and typhoid bacilli, the colon bacilli, the dysentery bacilli (such as the cholera vibrio), the *Brucella* (germs responsible for numerous human and animal infections, of which the best known is Malta fever), meningococci, gonococci (gonorrhoea agents), etc.

In order to extract an endotoxin from bacteria, numerous methods have been used. The simplest, if not the best, is the original one which was devised by Boivin in 1933. Briefly, this is how it works: the bacteria (typhoid bacilli, colon bacilli, etc) are placed for several hours in a refrigerator in the presence of a large amount of trichloracetic acid. This acid kills the germs, coagulates their proteins, and liberates into the medium in a soluble state their non-protein constituents. The preparation is then centrifuged, the precipitate is discarded, and the supernatant liquid dialysed in a cellophane bag, first in the presence of running water, then in distilled water. Through the pores of this bag the trichloracetic acid and substances of small molecular size originating from the germs are able to escape. The micromolecules, except for the proteins which have been rendered insoluble by the acid and are already eliminated by centrifuge, remain in the solution inside the bag. After several days the liquid is taken, slightly acidified, and treated with alcohol or acetone (added in large quantities). An abundant precipitate then forms which is separated by a new centrifugation. The precipitate is dried *in vacuo*, and the final preparation obtained represents the endotoxin.

This endotoxin, no matter what the germ

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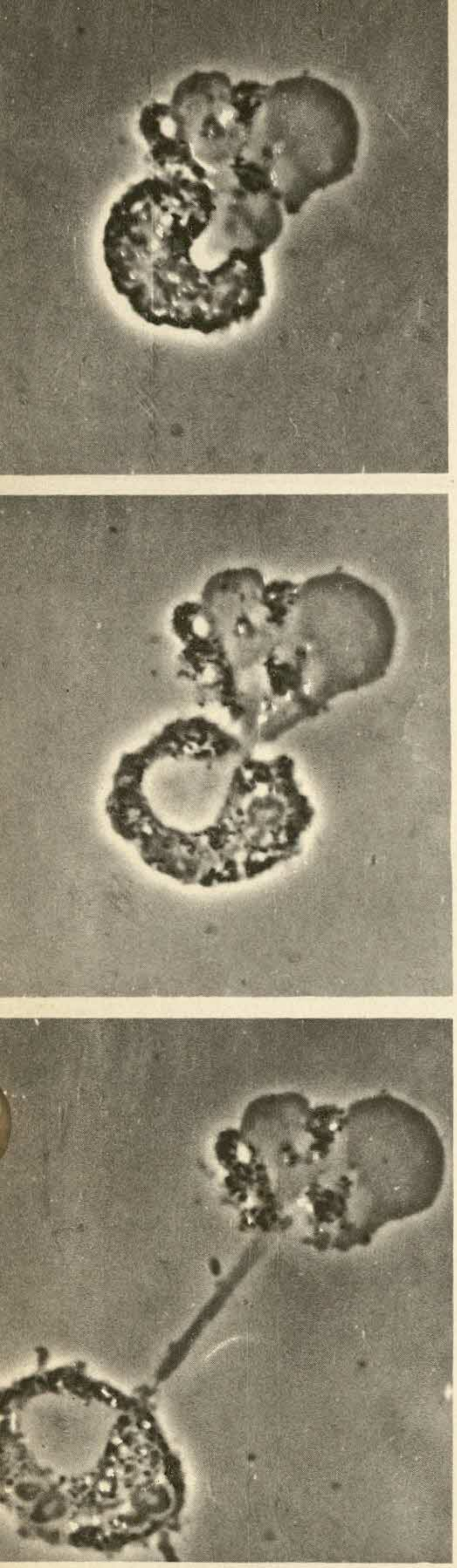
(1) *See the article by Dr van Heyningen on exotoxins.*



*Antibodies facilitate the ingestion of germs (dead or alive) or their fragments, by the phagocytes. Here a polymorphonucleocyte absorbs a fragment of cellular nucleus, treated with an anti-*DNA *antibody. (Mag.* × *2,500. Robineaux and Seligman).*

or the selected method of extraction, is found in the dry state to be a solid, almost colourless mass which dissolves easily in distilled water, giving an opalescent solution. Three types of substance comprise its structure: a polyoside (that is to say, a sugar substance), various phospholipids, and some polypeptides. For this reason the biochemists like to call these complexes glucido-lipido-polypeptides. They are justified in doing so; it is always necessary to remember that, in the present state of our knowledge, such a designation must not be considered as indicating a closely defined chemical complex. Each endotoxin, on the contrary, can be regarded as special, by the proportion of its fundamental constituents (polyosides, lipids, polypeptides) and also by the intimate constitution of each of them. The procedure of extraction itself, in any given strain, has an influence on this composition. Thus the method employing diethyleneglycol (Henderson and Morgan) and the method using trypsin (Raistrick and Topley) lead to complexes which are two or three times richer in polypeptides and two to four times poorer in phospholipids than the method using trichloracetic acid (Boivin).

Properties of endotoxins

A bacterial endotoxin invariably possesses two main physiological properties:

1. It is antigenic, that is to say, it is an antigen, which, as we have already seen, is a substance causing the organism receiving it to elaborate a new substance which can easily be demonstrated in the blood and is known as an antibody. In the case of endotoxins, the antibodies which appear have numerous properties, notably the ability to cluster into large masses (agglutination) the living or dead germs which harbour the corresponding endotoxin. They are also able to facilitate the absorption of the same germs by phagocytes (opsonization).

It is necessary to emphasize the importance of this last fact; for it is this which clarifies certain aspects of vaccination. Today, there is just cause for saying that the active fraction in Gram-negative germs currently used as a vaccine (for example, in anti-typhoid and anti-paratyphoid vaccination) is that which is represented by the endotoxin. This, among other things, has led to the proposal of a new synonym for the endotoxins, called antigen o. (Reasons of space forbid discussion of why the letter o was selected).

The antigenic power of an endotoxin is found only in the polyoside fraction. This fraction, however, is by itself only feebly antigenic, and we still do not know which of the other constituents of the complex, phospholipids or polypeptides, reinforce its action.

2. The second remarkable property of endotoxins is their toxicity. Cold-blooded animals are, in general, resistant; mammals, on the contrary, are receptive. The most sensitive animals appear to be mice, guinea pigs and rabbits.

From the point of view of immunology each endotoxin is specific in the sense that it gives rise to its own antibody. (Thus the antibody corresponding to typhoid endotoxin is immunologically different from that which corresponds to the gonococcal endotoxin.) Nothing comparable to this occurs in the pathological sphere; for, in fact, all the endotoxins, no matter from what germ they arise, produce identical reactions in the animal. When, for example, one injects a guinea pig or a mouse with typhoid or gonococcal endotoxin, the same state of intoxication takes place.

A strong dose, for example, 0·5 mg for a mouse of 20 gm or 2 mg for a guinea pig of 250 gm, introduced into the organism by the intravenous, intraperitoneal or subcutaneous route, is lethal. A short time after the injection the animal becomes drowsy and has diarrhoea, the respiration and heart-rate accelerate, and death finally follows within a few hours. At autopsy one is struck by the presence of diffuse haemorrhages in the viscera. When the injected dose has been a weaker one, the animal survives but its reactions are very rapid. The principal ones are profound changes in blood pressure, a rise in body temperature, hyperglycaemia and leucopenia.

The particular effect of endotoxins on temperature has given rise in the last few years to a vast literature, but this is hardly surprising, since the endotoxins are the most powerful pyrogens available to physiologists. In the affected animal (here we are thinking especially of the rabbit) hyperthermia manifests itself very early and continues for a certain length of time, the temperature returning to normal when the animal recovers. When this does not happen, the hyperthermia gradually gives way to a hypothermia. The cause of these fluctuations of temperature, in spite of much research, remains very largely a mystery; in particular, we do not know the part played by the central nervous system.

Leucopenia (reduction of the number of white cells in circulation) is generally accompanied by an inhibition of diapedesis, so that those white cells which are still present in the blood are no longer capable, as they are in the normal state, of passing through the endothelial walls of the capillaries.

An injection of endotoxin into the skin of a normal animal produces locally only discrete lesions. These become extremely large and haemorrhagic, however, when the first injection is followed twenty-four hours later by a second, if this is given intravenously. This special reaction has been given the name of the man who first described it: Shwartzman. Like the similar reaction of Sanarelli, it falls manifestly into the category of allergic reactions. Unfortunately, its fundamental nature is shrouded in obscurity.

In the hope of obtaining a better understanding of the mode of action of the endotoxins, various methods of increasing or diminishing the toxicity have been investigated. In general, toxicity can be increased by depriving the animal of its supra-renal glands, and it can, on the contrary, be diminished by the simultaneous administration of certain chemical products such as the sulphonamides, cortisone, and chlorpromazine. Nevertheless, the diminution obtained is always very weak and, in fact, the best prophylaxis is still vaccination. But even here there are many problems; for the phenomena of immunity (properly speaking, the intervention of an antibody) are associated with the phenomena of habituation, and it is impossible at the present time to establish the individual roles played in the process.

Biological interest of endotoxins

At the beginning of this article we said that the subject was of immense interest: why so?

1. Our first reply will astonish nobody. Bacteriologists were the first to interest themselves in endotoxins, in particular, those who were studying the constitution of the bacterial cell. Like all other cells, the bacterial cell has as its principal constituents, proteins, fats and carbohydrates. It is therefore in no way unusual by the nature of these constituents. What makes it unique and what in fact gives it its special character, is the manner in which these different constituents are joined to each other in order to give rise to complexes.

The bacteriologist will want to find out, to begin with, what characteristics are specific for each endotoxin; what it is, for example, that makes the typhus endotoxin different from that of the meningococcus in spite of an identical general 'arrangement'. This is a difficult task and it is becoming evident that only the bacteriologist working closely with the biochemist will be able eventually to inform us of the nature and the quantity of simple elements which contribute to the building-up of the whole.

2. After the bacteriologist and the biochemist, it is the immunologist who is absorbed by the problem. His task, also very difficult, is also concerned with the nature and mode of action of antibody and anti-endotoxin.

One question in particular is of paramount importance. Are these antibodies simply the trace of the passage of an endotoxin within the organism, or do they possess some special powers? The present evidence is that they can diminish the resistance of an organism to the microbe carrying the corresponding endotoxin by launching allergic reactions. More often, however, they increase it, on the one hand, by making the animal directly insensitive to the harmful effects of the poison and, on the other, by facilitating the phagocytosis of the living germs which carry the poison. This sort of action justifies, as we have already remarked, the use of certain vaccines such as the anti-typhoid and paratyphoid vaccine on a large scale.

3. What, in the meantime, is the pathologists' and the physiologists' interpretation of these phenomena? At the present time, it is they apparently who are asking the most interesting questions. Let us recall the special picture of intoxication to which the endotoxins give rise. We have described this as 'toxic shock' but this shock immediately brings to mind that designated clinically as operative shock or traumatic shock. Everything happens in this case as if the illness expressed less the effects of an agent normally foreign to our economy than the effects of stimuli having their origin within ourselves. Is this obscure? Let us explain.

An endotoxin spreads out naturally from the point where it has been artificially injected into an organism and a state of shock develops which manifests itself by leucopenia, circulatory troubles, fever and hyperglycaemia. Must we see in the different reactions a direct effect of the endotoxin? To all appearances, no. In reality, it seems that the toxin limits itself to setting in motion a series of inner mechanisms; this launching action has for the organism itself the value of a defensive reaction, but a defensive reaction which, because it exceeds its aim, becomes dangerous.

The sexuality of bacteria

Giulio A. MACCACARO

For a long time it was believed that the reproduction of bacteria was limited to the division of one element into two. The reality, revealed in these last few years by detailed studies, is much more complex. The ingenuity of research workers, with the help of some luck, which often plays a useful role, has thrown light on three possible mechanisms —transformation, transduction and recombination.

It is sufficient to recall that, from the general biological point of view, the word 'sex' designates above all the ability (anatomical and functional) which a male individual possesses of transmitting genes to another individual, the female, thus forming a new being of the species.

It was believed for a long time that bacteria do not possess this capability, so much so that the biologists always cited them as being the clearest example of strictly agametic reproduction.[1] But the microbiologists were not content with this idea and, on several occasions, they looked for, described, or even imagined the existence of sexual phenomena in bacteria.

For example, Ederlein noted in 1925 that certain granules, called gonidia, could be liberated from a bacterial cell, reproduce themselves, and be transformed into gametes by a true conjugation peculiar to them.

Lohnis, whose opinions were similar to those of Ederlein, had maintained in 1921 that direct union between individual cells was a frequent phenomenon; and Pothoff (1926) produced some specific examples with *Chromatium*, describing a tubular bridge between two conjugating cells.

Sherman and Wing, in 1937, attempted to induce hybridization between different bacterial colonies without obtaining any conclusive results (in 1906, Browning had already advanced similar views). Other experiments had been made sporadically, but always without success, until those of Gowen and Lincoln in 1942. All these setbacks proved that if there existed some phenomena like sex in bacteria, the current techniques did not permit them to be discovered.

It is to a young student (Joshua Lederberg) that the merit must go for having conquered these obstacles and overcome the difficulty in question, revealing ground that is wonderfully fertile in new data which, far from being exhausted, enrich the very foundations of biology.

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(1) Agametic reproduction: *reproduction which is carried out not by fusion of a male cell with a female cell, but by another process, as when one bacterial cell divides into two equal cells.*

(2) Genome: *chromosomic make-up.*

Genetic exchange in bacteria

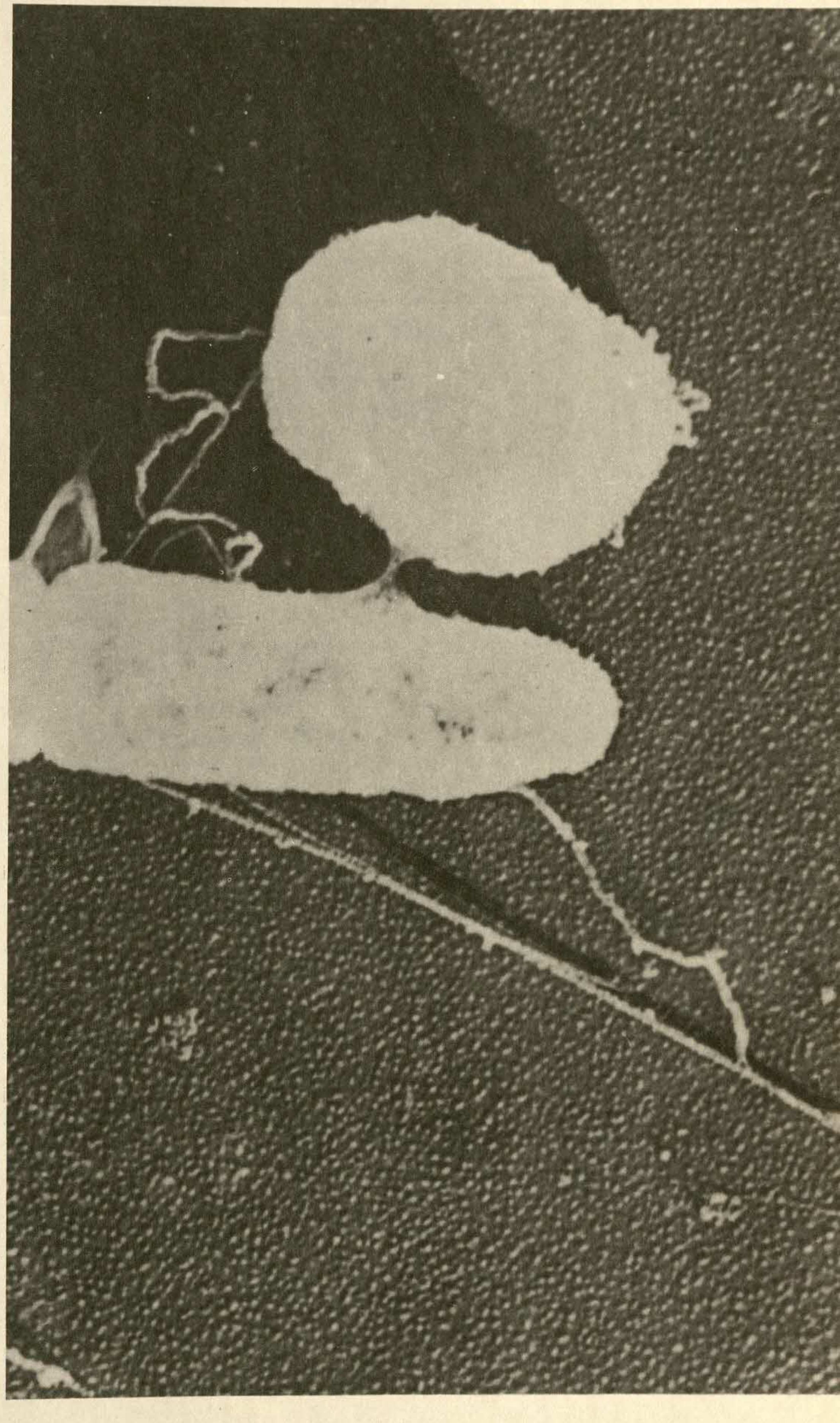
Amongst the higher organisms, whether animal or plant, sexual reproduction has the following significance: two genetic systems are able to mingle one with another and, in consequence of the reassembling of their parts, a new genotype is formed, capable of offering a suitable solution to the problem of the survival of the species. Under forms and behaviour whose complexity varies according to the evolutionary stage reached, this phenomenon consists, in the final analysis, of the fusion of two cells of different sex and the union of their genetic material.

It was long believed that the bacteria could not exchange genetic information in this way, but today we know not only that such an exchange takes place by the direct conjugation of two cells but also that bacteria possess two other means of genetic transfer apparently unknown elsewhere.

Genes and chromosomes are known to be made up of deoxyribonucleic acid (DNA) long macromolecules formed of two spirals entwined one upon the other. In the composition of these spirals molecules of phosphoric acid and pentose sugar alternate. The two spirals are like the two banisters of a spiral staircase. Between them are found the 'steps'. Each 'step' of DNA is made up of two substances, a purine base and a pyrimidine base. The purine bases are two in number, adenine and guanine; the pyrimidine bases are cytosine and thymine.

The order in which the different steps follow each other is subject to no rule; a molecule of adenine can be followed equally by one or other of the molecules we have named, and this can be followed in its turn by another molecule, and so on. But a strict law governs the construction of each step: if its purine part is composed of adenine, its pyrimidine part can be made up only of thymine, and *vice versa*. Guanine always pairs with cytosine.

Together, the four bases appear like an 'alphabet' formed of four symbols. Their succession along the length of the DNA helix, that is to say from step to step, spells 'words' written in this alphabetic fashion and deals with the process of cell-construction. Each word having a very special meaning is, in fact, what is normally called a gene.



The conjugation of bacteria leads to a transference of genetic material between the bacterial cells. (Photo Wollman).

The transference of genetic information from one cell to another is thus a transference of genes, and in the last resort, of a small quantity of DNA containing words and phrases and constituting an instruction to the cell to make specific substances.

A biologist with imagination, asked to formulate hypotheses as to reasonably simple processes by which this transfer can come to be carried out, could propose the following possibilities:

1. a small amount of DNA by itself may be able to move out of one cell and penetrate into another;

2. DNA may be transferred from one cell to the other by some vector which incorporates it;

3. two cells may draw near each other and be linked by a bridge of cytoplasm by which DNA moves from one cell into another.

Are these hypotheses too fantastic? Not for the microbiologist of today, who has demonstrated in the bacteria all three mechanisms of transfer which are now known respectively by the terms transformation, transduction and conjugation. In the rest of this chapter we shall discuss details and characteristics of these processes.

Transformation

Historically, the story of transformation starts with a germ, a mouse, and a false hypothesis.

The germ is the *Diplococcus pneumoniae* (or the pneumococcus). It is the aetiological agent of pneumonia and its individual cells are often surrounded by a polysaccharide capsule which varies in composition and in structure from strain to strain, but which remains constant within each strain. It is thus possible to distinguish different types of pneumococcus.

One says that a capsuled pneumococcus is in phase S and a pneumococcus without capsule is in phase R. The phase S is virulent for the mouse or, in other words, it can infect the animal and kills it if injected into it. The phase R is not virulent.

In 1928, Griffith inoculated some mice with killed germs in the virulent phase S of a type (III) of pneumococcus and with living germs in phase R of another type (II). The mice should have survived (as the germs which could kill them were dead and the living germs were not able to kill them). But they perished and from their bodies could be isolated some living germs in phase S of the type III pneumococcus.

Griffith believed that the cell of type II carried the ability to produce a capsule of type III and he supposed that the substances liberated by the killed germs favoured this synthesis. This hypothesis was false. Nevertheless, the experiment was among the most important in the history of microbiology. Indeed, several years later, other researchers demonstrated that an identical transformation could be produced equally well in a test-tube without the need to inoculate an animal with the killed virulent and live avirulent bacteria.

Later still, it was discovered that in place of the killed bacterial bodies from type III one could use simple bacterial extracts as transforming agents. In 1944, it was established beyond doubt that the active part of such a transforming agent was pure deoxyribonucleic acid, this same substance that is combined with protein as the principal constituent of the chromosomes in higher organisms (Avery, McLeod, McCarthy). This epoch-making discovery provided, for the first time, irrefutable proof that genetic information was transcribed and conserved in a definite chemical structure.

These experiments also showed not only that the transformation was effected by DNA without the intervention of other substances, but that it never reproduced itself spontaneously, becoming part of the heritage of all the cells which were formed from the transformed cell. Since then many more researches have been carried out on the processes of transformation and on the transforming agents themselves.

Although our knowledge is becoming greater day by day the number of species in which transformation has been demonstrated is still relatively restricted: it narrows down to *D. pneumoniae*, *H. influenzae*, *N. meningitidis*, with a very little in others, *B. subtilis*, and *Rhizobium. sp.*

Transformation of *E. coli* by DNA would have very special interest, for we have for this bacterial species, more than for any other, genetic data concerning the very different characters (see later); but the few results which seem to suggest the existence of transformation (Boivin and colleagues, 1945, Chargaff and colleagues, 1957) have not been confirmed in a convincing manner.

The apparent rarity of the phenomenon may be due to the fact that the cells of many bacteria produce large quantities of deoxyribonuclease—an enzyme which attacks and destroys DNA specifically—or possibly because the cellular envelopes (wall, membrane) in

(3) Mutagenesis: *induction of mutations due to the action of physical agents (ionizing radiations such as X-rays, alpha, beta and gamma rays, and non-ionizing radiations such as ultra-violet) or chemical agents (certain dye-stuffs, nitrous acid, abnormal purine and pyrimidine bases, etc).*

(4) Carcinogenesis: *induction of tumours by physical and chemical agents. Numerous carcinogenic agents are also mutagenic.*

(5) Phenotype: *the characteristics, as a whole, of an individual organism. It is the product of the interaction between its genotype—that is to say, its hereditary material—and the environment.*

(6) Mutant: *an individual in which one or several of its hereditary characteristics are modified in relation to its ancestry, after chemical modification of a gene.*

many species are impermeable to molecules as large as those of DNA.

The variety of characteristics which can be transferred from one cell to another by the process of transformation is very large. These characters go from the capsular antigens already mentioned to various enzymes, such as those which cause the fermentation of sugars, and to their power of resisting different anti-bacterial substances such as penicillin, streptomycin and sulphonamides.

This last example is particularly significant because bacteria which may never have been in contact with a toxic agent capable of killing them can, if treated with a DNA extract from another resistant cell, become resistant to this agent and transmit that resistance indefinitely to their offspring.

The process of transformation has not yet been fully elucidated. One knows that very few of the cells (0·01 to 0·20 per cent) coming into contact with DNA are transformed. This low frequency is certainly due to different causes and, entirely in the first instance, to the participation of little known factors, closely linked and capable of affecting the environment or the cells. Hotchkiss 1954 and Thomas 1955, showed that bacteria are permeable to foreign DNA for only a brief period of its division cycle.

Once present in the cell, the foreign particle of DNA becomes integrated within the host genome,[2] not by addition but by insertion. Characters are usually transmitted singly, suggesting that the transforming macromolecule is a fairly small fragment of the original genome. This fragment, nevertheless, must not be too small, since it can happen that transformation takes place for two characters at a time, presenting no physiological correlation between them (resistance to streptomycin and fermentation of a sugar).

The phenomenon of transformation is becoming of interest in other fields, such as in the study of mutagenesis[3] and of carcinogenesis,[4] both chemically and physically induced. The transforming substance is, as a matter of fact, sensitive to radiations and to substances which increase the frequency of mutation in the higher cells, both animal and plant.

Transduction

This specific type of genetic exchange can be considered as a particular case of the preceding. It is a question of a transformation effected by the mediation of a bacteriophage.

To understand the mechanism and its significance fully, it is necessary to recall certain general ideas concerning phages (which are treated in detail in another part of this work).

The phages are viruses which invade the bacterial cell, reproduce within them, and only in them, and then usually destroy or lyse the parent cell.

Phages that lyse cells are called virulent phages. Sometimes, however, other connections are established between the bacterial cell and the phage particle; the cell, having been infected by the phage, is not lysed, but, very much on the contrary, it reproduces itself normally and can go on doing so throughout an unlimited number of generations. During this process the phage is reproduced and transmitted from cell to cell. It may, however, regain its virulence in a certain number of cells, determining their lysis. The cell thus liberates other phage particles.

A bacterium housing a phage under these very peculiar conditions, almost symbiotic, is called lysogenic. The phage of such a cell is called a temperate phage and forms, properly speaking, part of the bacterial chromosome. But when it leaves the chromosome and becomes virulent, the phage carries away those fragments of a bacterial chromosome which are situated close to the point at which it was inserted. Thus the phage, infecting another cell, transfers to it some chromosome fragments of the cell from which it came.

The result from this is that the genes of a bacterial cell can be transferred and integrated within the genome of another cell, which will consequently modify the characters of those genes.

This mechanism of genetic transference carried out by the mediation of a bacterial phage is not exclusive or the most frequent. We have described it in detail because it illustrates in a remarkable way the relationships between the gene, the virus and the cell.

More frequently, it seems that the particles of the virulent bacteriophage—multiplying in the cell—embody by chance small segments of bacterial genetic material which they can transfer to other cells by infection.

Zinder and Lederberg, who in 1952 described this phenomenon for the first time, called the process transduction. First observed in *Salmonella*, transduction has now been verified in the micro-organisms of other genera such as *Escherichia*, *Shigella* and *Micrococcus*.

The particle of genetic material transferred by the phage possesses, in general, very limited properties. There are phages which carry away only a well-defined particle (the transduction therefore takes place always for

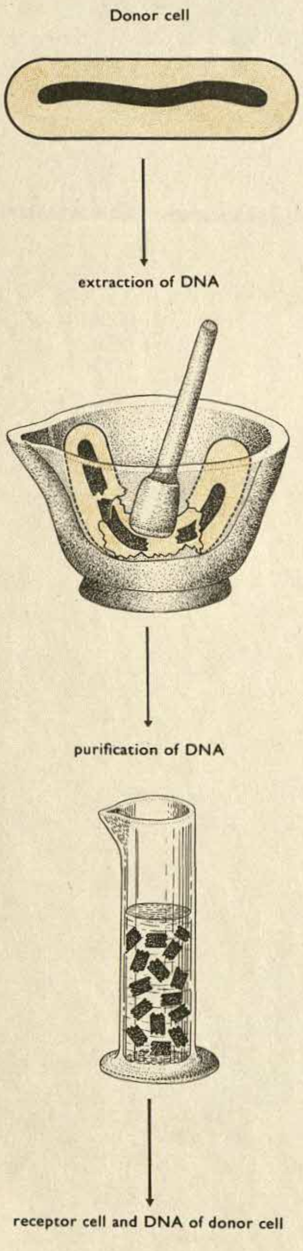
(7) Syntrophism: *relationship which is established between two cells whose capacities and biochemical deficiencies differ, and which can grow on a medium inadequate for either alone, by exchanging the metabolites which they need.*

(8) Ploidy: *the number peculiar to the reproduction of each chromosome in the cell. The cell is called diploid when each of its chromosomes is double and haploid when each chromosome is single.*

(9) Recombinant: *product of the combination between two mutants of the same strain.*

(10) Phenocopy: *individuals of which the phenotype corresponds to a genotype different from theirs. They generally reproduce under the influence of factors related to their surroundings.*

Transformation



the same characters). Other phages, on the contrary, transport different particles each time.

In the first case (limited transduction or special transduction), the phage—as we have seen—has a well-defined point of insertion in the bacterial chromosome, and yet it can function only as a vector of genes adjacent to this point. In the second case (unlimited or general transformation) the phage transports this or that gene with variable frequency.

The study of the simultaneous transduction of more than one gene has enabled their positions and the distances which separate them to be determined.

Evidently, the probability that two genes will be transferred together by a phage is all the greater if they are situated close to each other, and *vice versa.* If, for example, A and B are transferred at the same time more often than A and C, one could deduce that A is closer to B than to C. If, in addition, B and C are only rarely transferred together, one would be right to conclude that the probable order of the three genes is B, A, C. Thanks to this procedure and to more subtle methods than those we have outlined, it has been possible to carry out particularly delicate analyses of certain regions of the chromosome of *Escherichia* (Lennox, 1955) and of *Salmonella.*

Taking, for example, the regions of the chromosome bearing the genes controlling the various stages of cellular synthesis, it has been shown in particular instances that the order of the genes on the chromosome is the same as that of the stages of the synthesis (Demerec and colleagues, 1956).

If the chromosome fragment transferred by the phage does not become inserted into the chromosome but lives in the cell as a supernumerary gene, the transduction is then said to be 'abortive'. The supernumerary gene does not succeed in reproducing itself, but it does not remain without effect on the phenotype[5] of the cell which carries it. When the cell divides, the gene passes into one only of the two new cells, and so on for each generation. This transference of genetic material, which does not split into two and which affects only one cell per generation, is a unique example of unilinear heredity (Stocker, 1956). The study of this particular type of transduction eventually furnished extremely sensitive instruments for genetic analysis.

The mechanism of transformation is explained in the text. This diagram shows the experimental method used in its evidence. The DNA *extracted from the donor cell is incorporated into the chromosome of the receptor cell which, transformed thereby, becomes a cell with entirely new properties, according to the various subjects utilized.*

If it is true that transduction is in some way a particular type of transformation, it also reveals a new perspective in our understanding of virus-cell relationships. It represents, in a way, the first outline of sexuality in which the phage is a kind of bacterial gamete transmitting the genes from one cell to another.

Recombination

We have seen that transduction is only a preliminary of bacterial sexuality. At the time of this phenomenon of transduction, it produces not only a large transference of genes from one cell to another, but the participation of two cells in such an exchange attributes to the one a role of donor of genes (male) and to the other that of receiver (female).

As we have mentioned earlier, all the knowledge that we have on this matter arises from an experiment that Lederberg and Tatum made in 1946. To understand it let us recall that *E. coli* has a high capacity of synthesis, in that it can increase and multiply on a simple medium (called minimal) where nitrogen is provided by ammonium salts and where glucose is the only source of carbon and of energy. As the cell of *E. coli* contains substances of great complexity, such as amino acids, vitamins and nucleic acids, it is obvious that the cell is capable of making them by itself.

Let us indicate by the symbols A, B, C and D four of these substances, and let us mark by a plus or a minus sign, respectively, the ability or inability of the bacterial cell to synthesize them. For example, a given strain of *E. coli* is $A^+ B^+ C^+ D^+$, and from this one can obtain mutants[6] which have lost certain of these properties, for example, one mutant may be $A^- B^- C^+ D^+$ and another $A^+ B^+ C^- D^-$.

Lederberg and Tatum began their experiments by selecting mutants of these kinds. They then prepared three plates of 'minimal' medium. On the first of these they planted the mutant $A^- B^- C^+ D^+$; on the second, the mutant $A^+ B^+ C^- D^-$; and on the third, a mixture of the two. As one could reasonably expect, nothing grew on the first plate nor on the second plate, and nothing should have grown on the third. The third plate, however, showed a very small number (about one per million of seeded cells) of healthy colonies.

These colonies were evidently made up of cells A^+ B^+ C^+ D^+ and experiment showed that their descendants maintained this constitution in all later generations.

Lederberg thus excluded in this simple experiment certain alternative explanations. Growth was not a question of syntrophism,[7] because only a small minority of seeded cells formed colonies, nor was it caused by some transforming principles; and the mutants did not return to their original state by simple back mutation. For the first time (transduction was not yet known) the phenomenon of genetic exchanges between bacteria was demonstrated experimentally.

Lederberg and others confirmed that this exchange concerned especially the characteristics (A, B, C and D) for the matching of which a selective medium was used, and that it also affected an important number of other characters. The frequency of exchange for each of the latter enabled the order of the corresponding genes on the chromosomes of *E. coli* to be discovered. It was thus that genetics and microbiology met and initiated an unusual and very fruitful collaboration. One can cite among the first results the genetic analysis of bacterial resistance towards certain antibiotics (Cavalli and Maccacaro, 1951).

For some years the following model was considered valid: bacterial cells have one chromosome, that is to say, they are haploid.[8] Occasionally two can merge and form a diploid (binucleate) cell where some of the phenomena of recombination are produced. If one chromosome is A B C D E F and the other is a b c d e f, segments may exchange to form a b c D E F, or A B C d e f, etc. However, the diploid cell is unstable, and it very quickly gives rise to two new haploid cells. In each of these, the characters, that is to say the genes, among those which are the parent ones, will be there and sorted in a new way. Such a model would explain the facts described and would attribute practically the same role to each of the two conjugated cells.

An English microbiologist, Hayes (1952), studied the effect of treatments capable of injuring one or other of the two cells (ultraviolet rays, streptomycin) on the frequency of recombination, and discovered that the integrity of one member of the union was much more important than that of the other in the genetic success of the conjugation. One of the cells participates only with its genes, the other participates with its genes and also with all its cytoplasm in the constitution of the new cell. Hayes was thus the first to put, in explicit and experimental terms, the problem of the genetic exchange of bacteria as being either reciprocal or effected in a single direction. This subject was later developed by the French microbiologists Jacob and Wollman, and by Hayes himself.

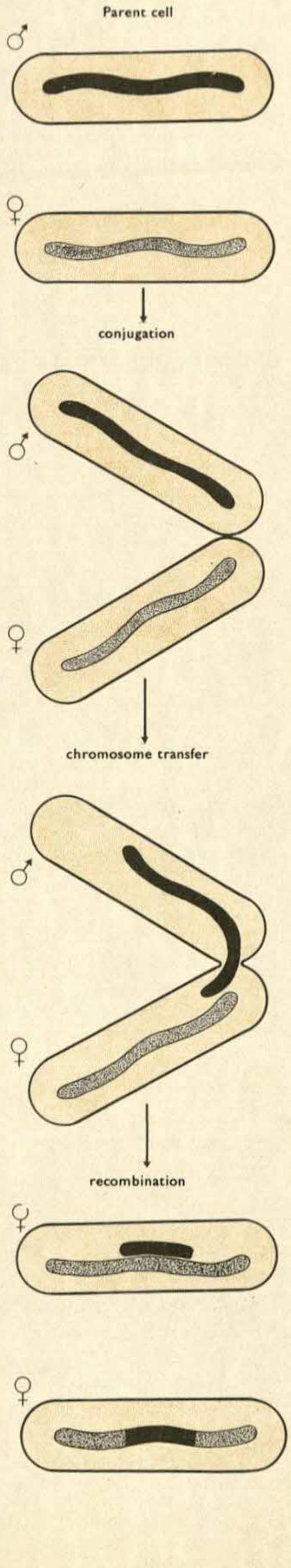
In the meantime, another important discovery had been made. It was made independently in three countries in 1952, by Cavalli in Italy, Lederberg in the United States and Hayes in Great Britain. These researchers observed that the cells of *E. coli* are not all capable of conjugation with other cells. Two types were distinguished, F^+ and F^- (F designates fertility), that are defined thus: $F^- \times F^-$ gives non-fertile crossings; $F^+ \times F^+$ gives crossings with relatively poor fertility; $F^+ \times F^-$ gives much more fertile crossings. It was also noticed that the cells F^- and F^+, if grown on the same medium, always gave F^+ descendants; and that in the $F^+ \times F^-$ cross the genetic contribution of the two parent cells was unequal, the hybrid always having much more of the characteristics of the F^- parent than of the F^+ parent. Thus, two sexual types of bacteria appear more and more clearly: the female F^- cell, incapable of crossing with other cells of the same sex, and contributing with all its cytoplasm and its genetic make-up to the constitution of the recombinant,[9] receives some genes from the male F^+ cell (in general receiving only one part of the genome of the male F^+ cell).

The two types of cell can also be distinguished by properties of their surfaces, thus indicating the site of the phenotype expression of bacterial sex.

There exists yet another type of male cell. Cavalli in 1948, and later Hayes, found by chance that some mutants of *E. coli* crossed with others of F^- type gave rise to descendants a hundred to a thousand times more fertile than usual.

These strange types have received the name Hfr (high frequency recombination). They represented for a while a difficult problem. Cells of this type recombine as F^+ cells: they have the same phenotype characteristics, they are derived from F^+ strains and can return to the F^+ state, but they certainly do not contain the F factor in their cytoplasm,

Recombination



Not only is a large transference of genes from one cell to another accomplished by the mechanism of recombination, but also the two cells participating in this exchange behave as male (donor of genes) and female (receiver of genes).

for they can neither use it to infect F^- cells nor transmit it to their descendants. By studying the kinetics of crossings with F^- strains Jacob, Wollman and Hayes succeeded by means of a series of particularly fortunate and well-thought-out experiments in directing the whole problem of bacterial sex towards the most convincing solution that has been advanced up to date.

They found means of interrupting a cross in its successive phases and of determining the number and type of recombinants which could be attributed to each phase. They noted that:

1. the transference of genes of the cell Hfr (male) to the cell F^- (female) takes place in such a manner that a given Hfr strain always transfers genes in a set order, first the gene A, then the gene B, then the gene C, and so on;
2. the order of transference is related to the order which the genes occupy on the chromosome;
3. the time taken between transference of one gene and another is proportional to the distance between them on the chromosome.

These results show that, contrary to what was believed for some time, conjugation concerns only the transference of the chromosome, this passing into the female cell, beginning always with the same gene and continuing in the same direction. White, Jacob and Wollman have made three other important discoveries relating to differences between strains:

1. the Hfr chromosome does not always begin with the same genes;
2. at all times the order remains unchanged, even if
3. for certain strains the order is exactly the reverse.

Thus for the sequence of genes A B C D E F G, there are, for example, some Hfr strains with chromosomes C D E F G A B or D C B A G F E, but there is never a chromosome B C D G A E F.

It is difficult to interpret this result in a simple and coherent manner unless the hypothesis is accepted that the chromosome of *E. coli* is in fact circular, and that the different types of Hfr chromosome have at numerous points on this circle openings with different possibilities for transference. Wollman, Jacob and Hayes believe this to be true and believe that the genetic material of the F^- cell is organized in a circular chromosome which divides into two, and as such transmits itself to descendant cells. The F^- cell can be infected by other cells which contain F, and itself become F^+.

It happens that in certain F^+ cells a particle of F substance leaves the cytoplasm and inserts itself at a certain point on the chromosome. At this point the circular chromosome breaks and becomes a filament with two free extremities; that which remains attached to F is the tail, and the other is the head. The transference of the chromosome during crossing is accomplished from head to tail. When F fixes itself to the chromosome, it disappears from the cytoplasm, so that an immunity very similar to that which is produced between the lysogenic germ and the moderate phage is established. (See above: Transduction.)

The true and proper crossing is thus that which occurs between a Hfr male and a F^- female. In the crossing $F^+ \times F^-$, the effective males represent in fact a small percentage of Hfr mutants that have arisen from F^+ cells.

There exists also among these latter numerous phenocopies[10] of F^- which cause the low fertility of the $F^+ \times F^+$ crossing.

Convincing experimental results support all the details of this model and at the moment there are none incompatible with it. In a few years' time other experiments and observations may require the formulation of other hypotheses of bacterial sexuality. Today, there is good reason for accepting the scheme which we owe to Jacob, Wollman and Hayes.

The importance of a scientific discovery must be measured according to the portion of truth it contains and not according to the applications, more or less immediate, to which it lends itself. We shall not ask ourselves therefore what are the fruits brought by bacterial genetics during the brilliant first years of its life. We shall recall only that this new discipline has contributed to the clarification of problems related to the production of anti-bacterial drugs, to the more rational use of these drugs, and to the way in which pathogenic germs adjust themselves to their action.

The modern methods of analysis of bacterial variability also allow us to understand the bacteria much better than heretofore, even those properties such as virulence, pathogenicity and production of toxins, and to devise new methods for improving industrial fermentations.

Meanwhile, it seems to us more important to record the contribution of bacterial genetics to general biology, the science whose progress governs, in the final resort, that of applied biology.

HISTORICAL NOTES

1906–1946
Numerous researchers postulate that bacteria have some form of sexual reproduction more or less similar to that of lower plants.

1928
GRIFFITH *carries out the first experiment on the transformation of one type of pneumococcus into another by putting the first in contact with some killed cells of the second.*

1944
AVERY, McLEOD *and* McCARTHY *show that the transforming principle of Griffith is pure desoxyribonucleic acid.*

1948
LEDERBERG *and* TATUM *demonstrate genetic recombination in* E. coli.

1952
ZINDER *and* LEDERBERG *discover transduction in* Salmonella.

1952
CAVALLI, LEDERBERG *and* HAYES *demonstrate independently the existence of a sexual differentiation in* E. coli.

1956–7
JACOB *and* WOLLMAN *propose the model of the circular chromosome and of the mechanism of sexual differentiation in* E. coli.

The study of mutations

Paul MANDEL

The favourite experimental material of geneticists was for a long time the fruit fly (drosophila). Today equal interest is centred on the microbe. This, like all living beings, has an hereditary mechanism which helps it to survive in its reproduction. The mechanism of genetic reproduction is marked by great regularity, and only from time to time is this regularity broken: it is then one comes up against mutation (the loss or acquisition of a new character).

In recent years the study of bacterial mutations has given rise to research of immense interest: we now know how to induce mutations almost at will, and can identify their chemical substrate. The primordial role of deoxyribonucleic acid (DNA) has also been revealed.

By bacterial mutation is meant a sudden and permanent modification which affects one or more characters of a bacterium and which is transmitted by heredity. Such modifications were observed in the nineteenth century, but it is only during the last twenty years that important advances have been made in their study.

All micro-organisms possess an hereditary mechanism which is reproduced at each cellular division.[1]

Today bacteria constitute the favourite material for studying mutations and the control of their hereditary character. This is because very large populations of bacteria can be easily handled in the laboratory. Even if we assume that bacterial mutations are as rare as those of multicellular beings, their discovery is easier. Because it divides so rapidly, a single bacterium after a day's incubation provides 10^7-10^9 cells. It is important not to confuse mutations with morphological or metabolic changes in harmony with the environment and which themselves are not hereditary[2] (fig. 1).

To detect mutations it is necessary to show new characteristics in cells either within a culture or within a colony. If the mutants are in a high proportion, and are accompanied by a change in the appearance of their colonies on a solid medium, they will be easily revealed. The proportion of spontaneous mutations is small, 1 in 10^7-10^8, and even when mutations are induced this may be raised to only 1-2 in 10.[3] Provision of evidence is difficult, and imposes the use of methods or agents which favour the growth of the mutant in relation to the original cells.

For example, let us consider Lederberg's method applied to mutants which have lost the ability to synthesize a compound. The first stage of this method consists of the simultaneous incubation of the original strain (called wild or prototrophic) and micro-organisms deprived of enzymatic equipment, to produce a constituent that is auxotrophic. The appearance of this auxotrophic constituent is induced in a Petri dish on a plate of agar containing a rich nutritive medium (meat extract, peptone, etc). After a few hours, a disc of sterile velvet is fixed on an aluminium cylinder and applied first to the surface of the culture, and then to a plate of virgin agar containing a poor medium (glucose, an ammonium salt, phosphate and other mineral salts).

In this fashion one seeds a faithful replica and true impression of the initial culture on to the fresh plate. After incubation the colony patterns on the two plates are compared. On the rich medium both phototrophic and auxotrophic colonies will appear. On the replica certain colonies will be absent; these correspond to the auxotrophic mutants which cannot, in this poor medium, find the compound they need for their development, and have lost the power of making it.[3]

(1) *This mechanism is present in each cell and represents the genotype, that is to say, the hereditary foundation to be distinguished from the phenotype, made up of the collection of characters accessible to direct observation and which show the reactions of the genotype to the environment.*

(2) *This is the case in certain adaptive enzymes. The presence of lactose in the culture medium can give rise to the appearance of lactase in the bacterial cell. But this will appear in descendants only in the presence of lactose. Thus the ability to hydrolyze lactose is not hereditary. The transmissible character is the ability to produce the enzymes in the presence of the corresponding substrate.*

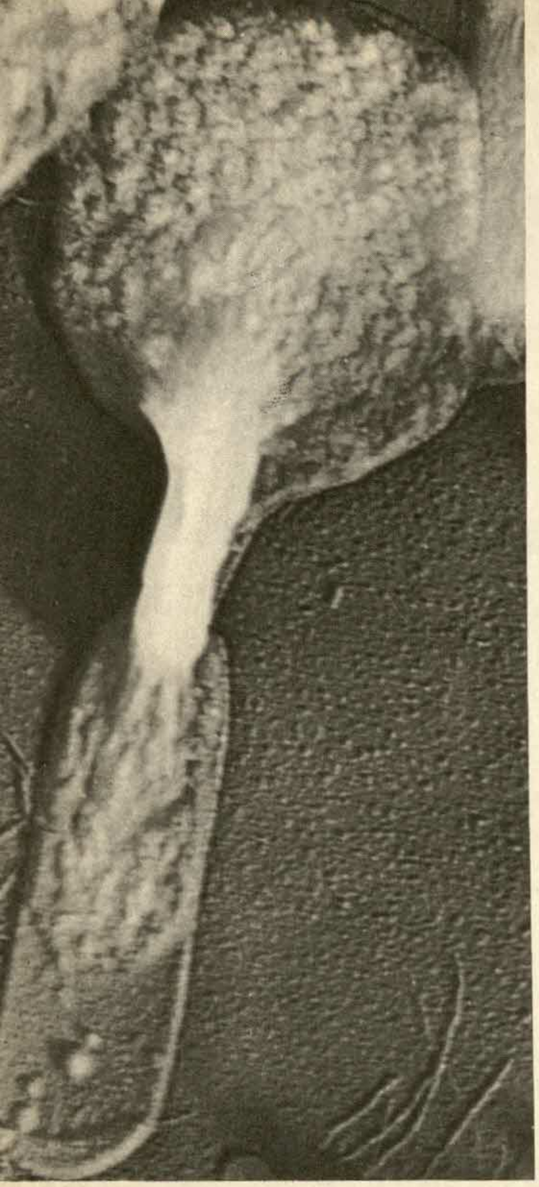


Fig. 1 Bacteria provide the biologist with valuable material for the study of mutations. These latter are just as rare as in multi-cellular organisms but are nevertheless easier to reveal because of the high frequency of bacterial multiplication. Here is seen a process of asymmetrical division of the bacillus of Morgan (mag. × 38,000 Instituto Superiore della Sanità, Rome).

Glutamic acid $\xrightarrow{1}$ acetylglutamic acid $\xrightarrow{2}$ semialdehyde of acetylglutamic acid $\xrightarrow{3}$ N-acetylornithine $\xrightarrow{4}$ ornithine $\xrightarrow{5}$ citrulline $\xrightarrow{6}$ arginine

Fig. 2 The synthesis of arginine

The estimate of the rate of mutations is of obvious interest, in that it concerns both spontaneous and induced mutations; various methods have been put forward for establishing this rate.

The various types of mutation

(a) *Mutations characterized by a resistance to chemical agents, and in particular to antibiotics*

Certain mutants can grow normally in the presence of a concentration of 1,000 μg/ml of the antibiotic streptomycin, whereas the original strain is inhibited by 10 μg/ml. In general, such mutants retain sensitivity to other antibiotics to which the parent cell is susceptible, but in certain cases simultaneous resistance appears in two antibiotics having a similar structure, such as aureomycin and terramycin. The interest in mutants resistant to antibiotics is obvious in the medical field. Their appearance during infection or their contagious transmission makes therapy difficult, because the antibiotic to which the bacteria has become resistant is of no use.

(b) *Mutations by loss of an enzymatic function*

The study of mutations in which an enzymatic function is lost (expressed by the inability of the mutant to synthesize a compound) has led to many important discoveries in physiology, bacterial genetics and biochemistry.

If bacterial colonies growing on a solid medium containing all factors necessary for growth, in particular all the amino acids, are compared, using the replica plating technique, with those on a minimal medium, colonies will appear on the rich medium which do not appear on the poor medium. If we now supplement this medium with arginine, certain of these colonies may be able to grow. It follows that such colonies are composed of mutants which have lost the ability to synthesize arginine. But this amino acid is known to be formed by a series of reactions as represented in fig. 2, and it suffices for only one of these to be blocked to prevent the production of the final compound, arginine. Thus, in order to fix the character of a mutation more precisely, all that is required is to determine which among the components in fig. 2 is necessary and adequate for the resumption of growth. The deficient enzymatic equipment is that which is responsible for the reaction immediately preceding the formation of the precursor in question. With the aid of a series of mutants incapable of synthetizing a constituent, it should be possible to say for certain whether a supposed precursor is the effective one. The renewal of growth of a mutant, with this compound present, will furnish proof of it. By using this technique, much light has been shed on the processes of biosynthesis of numerous amino acids.

(c) *Mutations by change in immunological type*

Among these mutations one merits particular attention, namely the variation which is accompanied by changes in the appearance of colonies from a smooth to a granular texture: S (smooth)→R (rough). This change is accompanied by the disappearance of an antigen characteristic for the surface of cells of type S and, in pathogenic species, by a complete or partial loss of virulence. The absence of all synthetic capacity for the antigenic substance S corresponds to a mutation characterized by the loss of an enzymatic equipment. Modifications of the appearance of colonies imply other changes at the bacterial surface. The spontaneous variation S→R often arises under conditions of normal culture, but can also be induced. For example, a strain of pneumococcus grown in the presence of an anti-serum specific for its type of capsular polysaccharide, can give rise to the appearance of the R form. Even the simple addition of certain mineral salts to the culture medium can promote this variation. Some intermediate states between S and R forms are possible. The reversion R→S appears very rarely under usual conditions of culture.

(d) *Mutants resistant to the lytic action of bacteriophages*

Among the descendants of a strain of colon bacilli lysed by bacteriophages, colonies resistant to one or several types of phage can be seen to appear. The mechanism whereby resistance is acquired can be of a diverse nature; for example, it may depend upon the disappearance of the property of adsorbing the phage on the bacterial cell.

(e) *Mutations concerned with chromogenesis*

The capacity many bacteria have of producing pigments is also subject to mutation, either independently or in a direction parallel to other characters. This type of mutation is easier to reveal than others.

(f) *Mutations characterized by the acquisition of an enzymatic function, real or potential*

Certain strains of *E. coli,* incapable of fermenting lactose, produce mutants with this property. It is often necessary to induce this mutation by the presence of lactose, which makes the requisite adaptive enzyme appear.

Experimental mutations

(a) *Non-specific induction*

Ultra-violet and x-rays are highly lethal for bacteria, and provoke a considerable increase in the rate of mutations among the surviving cells. Numerous types of mutation can thus be induced, such as resistance to bacteriophage, loss of the ability to synthesize a compound or enzyme, etc. Mutations can also be produced by chemical agents, alcohol esters, manganese salts, nitrous acid, and nitrogen mustards. The presence of oxygen increases the number of mutations obtained by radiation. Furthermore, the addition to the culture medium of peroxides, amino acids or nucleotides treated with peroxides, increases the rate of mutations.

(b) *Specific induction by the transference of genetic material*

It has been shown in an earlier chapter[4] that new bacterial types appear following conjugation, with the passage of an hereditary mechanism from one cell to another. It is appropriate to examine here the transformations of the bacterial genotype due to products of bacterial origin. Griffith, in 1928, injected mice with cultures of the non-virulent R form of the pneumococcus and of heat-killed S pneumococci, and isolated living and virulent S-form cells. It was deduced from this that the material arising from the S forms can impose its own hereditary characteristics on the R forms. Avery and McCarthy later (1944) isolated from a type S pneumococcus a highly polymerized deoxyribonucleic acid (DNA) which produces the R→S transformation. Non-virulent intermediate forms with R colonies can appear in the course of such experiments. It was thus demonstrated that the DNA of one strain can impose its own hereditary characteristics on bacteria of another strain.

Resistance to antibiotics such as penicillin and streptomycin has been obtained by Hotchkiss by growing sensitive strains in the presence of DNA from resistant strains; these resistant forms are comparable to those which have been observed spontaneously. The capsular specificity of the *Hæmophilus influenzae* has been transformed by the DNA of a mixed type. Recently, some interspecific transformations have been recorded between *H. influenzae* and *H. parainfluenzae* by the use of DNA from one of the two (Schaeffer, Alexander and Leidy). Furthermore, some transformations of the antigenic property of bacteria by a lysing agent of a near colony have also been reported for *E. coli* by Boivin and colleagues, and for other bacteria without the active agent having been defined in this case.

A particular type of transfer of genetic material, called transduction, is also discussed in an earlier chapter. Here genetic material is transferred from one bacterium to another by a bacteriophage which takes with it a portion of genetic material of one bacterium and places it in another cell, which thus undergoes a specific transformation. Other means of transference of genetic material may also exist.

The mechanism of mutations

Knowledge of the mechanism of spontaneous or induced mutations involves the making evident of the chemical and physical phenomena concerned. But before tackling these problems, it is advisable to define the biochemical nature of the base of hereditary properties; then to try to find by what changes in this biochemical base mutations appear, and according to what method the changes are transmitted to descendants.

That the principal constituents of genes are deoxyribonucleoproteins[5] was first suggested by Schulz in 1941, on the basis of his work with Caspersson. It has since been confirmed that the maximum frequency of mutations obtained by ultra-violet rays coincides with the zone of maximum ultra-violet absorption by DNA; and that the zones considered as the loci of the genes coincide with a high concentration of DNA (observation made by microscopy in the ultra-violet). The role of DNA in the production of mutations and in transformation has already been mentioned; deoxyribonuclease, an enzyme which breaks down DNA, destroys the power of transformation. In growing donor micro-organisms on radioactive phosphorus, the radioactivity of their DNA is found again in the genetic material of the recipient cells. In spite of all this evidence it nevertheless must be borne in mind that other substances may participate in the transmission of hereditary characteristics.

It is accepted that DNA is made up of two parallel chains of nucleotides, winding themselves spirally around a common axis and united by hydrogen ions (hydrogen link). The two chains are complementary and, when unrolled, have the appearance shown in fig. 3, with the deoxyribonucleotides having adenine

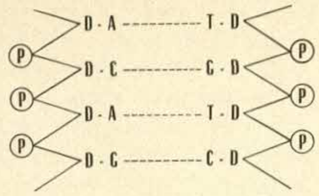


Fig. 3 Diagram of a portion of the DNA *molecule according to Crick. P: phosphoric acid; D: deoxyribose; A: adenine; C: cytosine; G: guanine; T: thymine; . . . hydrogen link.*

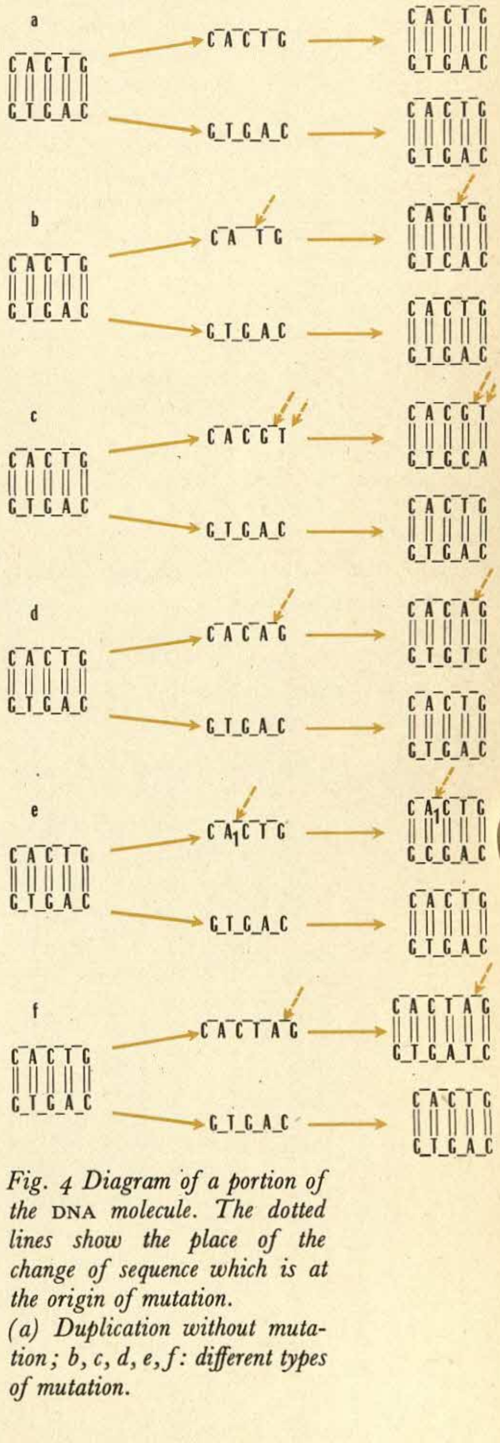


Fig. 4 Diagram of a portion of the DNA *molecule. The dotted lines show the place of the change of sequence which is at the origin of mutation.*
(a) Duplication without mutation; b, c, d, e, f: different types of mutation.

for their nitrogen base, always lying opposite a deoxyribonucleotide containing thymine. Similarly the nucleotide with guanine is paired with that containing cytosine. In the course of cellular and nuclear division the two chains of the helix separate; opposite each is formed the complementary chain, as indicated in fig. 4a. It is now believed that a gene corresponds to a certain number of paired nucleotides which follow a specific sequence in their nitrogen bases.

It was believed at first that radiations might produce mutations in damaging the structure of the chromosome by rupture, or by destruction of an exact site in the DNA molecule. But although these hypotheses may merit retention, numerous observations show that the phenomena associated with mutation are more complex. Thus, the inhibition of protein synthesis by chloramphenicol during a period immediately following ultra-violet irradiation, considerably reduces the number of mutants. The enrichment of the medium with purine and pyrimidine bases before irradiation favours the appearance of mutants. The increase in the rate of mutations is correlated with the intensity of nuclear synthesis by the bacteria in the period which immediately follows irradiation. As it was observed that an accumulation of the precursors of nucleic acids follows irradiation, one might ask what role these precursors play in the appearance of mutations.

It has also been proved that agents directly modifying the purine or pyrimidine bases can give rise to mutations. This is the case with nitrous acid, which produces breakdown, and very probably also with certain alcohol esters. The incorporation of a structural analogue[6] of a nucleotide base in the DNA of certain bacteriophages leads to an increased production of mutants.

Taking account of these facts and of the constitution of DNA, various hypotheses have been formulated to explain the appearance of a mutation at the molecular level.

(a) *Partial or total destruction of a base of one of the two polynucleotide chains which enter the helix*

The replacement of the destroyed DNA by another active base modifies the sequence of one of the chains and consequently that of the complementary chain and of the helix (fig. 4b).

(b) *Rearrangement of the bases leading to a modification of the nucleotide sequence of the* DNA *molecule (fig. 4c)*

(c) *Replacement of a base of one chain by another at the level of one or more nucleotides*

The complementary chain which is formed opposite the first is equally modified (fig. 4d).

(d) *Tautomeric change of the normal form of adenine (amine A) into the form amine A_1*

A_1 should combine in the complementary chain with a molecule of cytosine. After a division, the form A_1 should return to the form A, but a mutation has already been produced in the complementary chain (fig. 4e). A similar situation can be produced for thymine, whose tautomeric form should combine with guanine.

(e) *The introduction, in the course of* DNA *synthesis, of a supplementary nucleotide into one of the chains*

This would be followed by a change in the structure of the double helix. In this latter case various modalities can be envisaged (fig. 4f).

Every possibility results in a change in the nucleotide sequence of the polynucleotidic chains of DNA which, according to current ideas, provides the code for hereditary characteristics. The examples b, c, d, e, and f are caused by a metabolic error or fault of biochemical control of the synthesis of DNA during its repetition. The frequency of the error increases in a period following ultra-violet or X-irradiation by the accumulation of precursors and the stimulation of nucleic acid synthesis. This has been verified by using radioactive tracers in which the daughter cell of a mutant can be shown to contain half of the double helix coming from the mother cell, the other being made up of new material.

The ribonucleotide precursors of ribonucleic acid take part in the metabolism of carbohydrates, fats and proteins. It has been shown that routes exist for the conversion of ribonucleotide to deoxyribonucleotide. Under the influence of an important change in metabolic activity, a particularly important quantity of a ribonucleotide may be removed from the common pool, leading to disequilibrium in the distribution of precursors; and this may make possible an error in the synthesis of DNA. Note that there is a certain parallelism between the disturbances in the precursors and the frequency of mutations. These results encourage the continuance of research in this field; and the above hypothesis—and it is only a hypothesis—will need to be proved by irrefutable experiments.

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(3) *These transferred colonies are absent from the poor medium, but on the rich medium they can be counted and isolated by re-incubation in appropriate conditions. For the isolation of auxotrophic mutants Davis proposed using penicillin, which in a poor medium kills the growing cells, that is to say, the prototrophes which grow well, so that the deficient mutants (which because of this fact cannot multiply) can be re-inoculated and cultivated on solid media more or less complete without penicillin. It is easy to demonstrate a mutant's resistance to an antibiotic or to certain bacteriophages. The antibiotic or bacteriophage is introduced into the solid culture medium and the number of colonies that form are counted.*

(4) *See the chapter on 'The Sexuality of Bacteria'.*

(5) *A deoxyribonucleotide or nucleotide of* DNA *is made up of a molecule of phosphoric acid, a molecule of sugar (deoxyribose) and a purine base (adenine A or guanine G), or a pyrimidine base (thymine T or cytosine C). The nucleotides join each other through the phosphoric acid molecule forming polynucleotide chains of* DNA. *The deoxyribonucleoproteins result from the association of specific protein molecules and* DNA.

(6) *The* structural analogues *of the purine and pyrimidine bases are substances obtained by chemical synthesis, the composition of which differs in detail from that of normal bases. As they are similar to the latter, they can be incorporated in the nucleotides of* DNA *in the course of biosynthesis.*

The L forms

Paul HAUDUROY

L forms of bacteria are peculiar in their appearance, size and method of reproduction. Sometimes, a fluke in a bacteriological scrutiny will reveal their existence, but more often they are obtained by subjecting normal bacteria to the action of various physical or chemical agents. Certain L forms exist only in a transitory state; when the medium in which they live becomes suitable, they return to their usual form. Others remain permanently as L forms. Today these L forms evoke great interest.

In 1925, Levaditi, Nicolau and Poincloux discovered in pathological specimens a germ which they named *Streptobacillus moniliformis* (since called *Haverhillia moniliformis*). In 1935, Mrs Klieneberger-Nobel, examining cultures of this microbe on a solid medium, noticed side by side with normal colonies other scarcely visible punctiform colonies composed of small grains of varying shape (fig. 1), which she succeeded in cultivating. In 1947, she rediscovered similar bodies in the neighbourhood of normal colonies of another microbe, *Spherophorus necrophorus*.[1] They did not resemble any known bacteria. From where could they have come? Were they a new form of microscopic life multiplying by the side of normal bacteria and living in symbiosis with them? (Several examples of symbiosis are known in bacteriology.) Another hypothesis was that L forms are a 'modification', as yet unknown in the evolution of normal bacteria and a phase in a cycle itself unknown. Mrs Klieneberger-Nobel favoured the first hypothesis. She called these 'forms'—of which she still knew very little—'L forms', after the Lister Institute in London where she was working. Her strange discovery attracted the attention of numerous researchers and was confirmed for other microbes such as *Escherichia coli* and *Hæmophilus pertussis*.

American research workers—in particular Dienes, alone or in collaboration with Smith—discovered that the L formations of Klieneberger-Nobel can abandon the primitive form by which they are recognized and in certain conditions can change into perfectly normal bacteria. It was no longer a question of bacterial symbiosis. The second hypothesis conforms better with the facts: the L forms were a 'phase' in a bacterial cycle about which

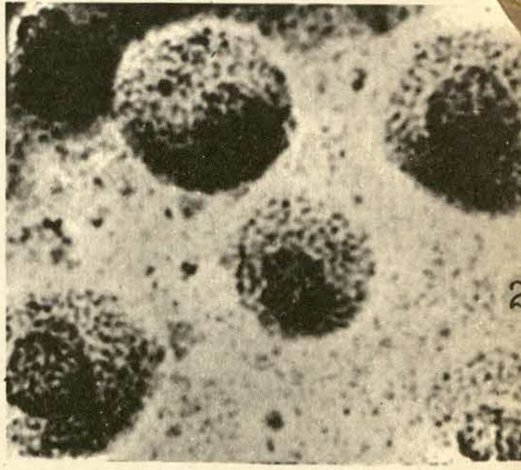

Fig. 1. L forms are composed of small granules of varying shape. Note that the centre is much more dense than is the periphery. (After Tulasne.)

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(1) *For the technical terms, refer to the glossary on page 44.*

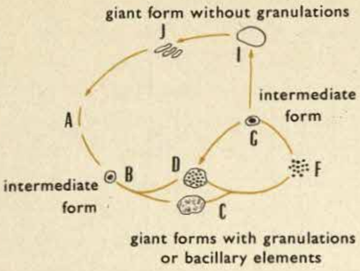
giant form without granulations
J
I
intermediate form
A
G
D
F
intermediate form
B
C
giant forms with granulations or bacillary elements

Fig. 2 Cycles of development of L *forms (see text for explanations).*

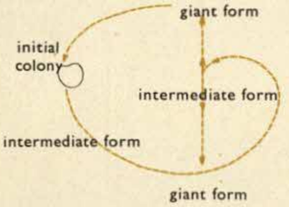

Fig. 3. Observable development of colonies of L *forms, visible to the naked eye.*

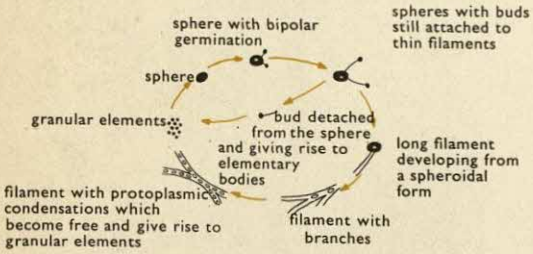
sphere with bipolar germination
spheres with buds still attached to thin filaments
sphere
granular elements
bud detached from the sphere and giving rise to elementary bodies
long filament developing from a spheroidal form
filament with protoplasmic condensations which become free and give rise to granular elements
filament with branches

Fig. 4 Development of an organism of pleuro-pneumonia after recent isolation (external cycle) or conservation in a laboratory (internal cycle). (After Wilson and Miles, 1955.)

nothing was known.

Up to this time, observations similar to those of Klieneberger-Nobel and of Dienes were rather infrequently reported. They seemed to require both very close study and a fortunate chance; it was impossible to obtain them at will.

In 1942, Mrs Pierce discovered that penicillin (added to culture media in suitable amounts so that microbes continued to develop perfectly normally) favoured the appearance of L forms. By this procedure it is possible to obtain L forms easily, and here again such forms can sometimes revert to the normal bacteria from which they arose. Thanks to this technique large numbers of bacterial species have been examined, and many positive results have been obtained. However, microbes kept for a long time in a laboratory and subcultured many times do not give rise to L forms.

Other antibiotics have also been tried and their action is irregular; for example, streptomycin is valueless whereas chloromycetin and aureomycin are exceptionally favourable. A great many other chemical and physical agents were next examined, such as calcium, lithium and cadmium salts, glycerol, organic products of bacterial growth, glycine, caffeine, high concentrations of raffinose, physical agents (cold, x-rays), biological products (antibacterial serum complex-complement, bacteriophage), etc. Many of these affected L formation to a greater or less degree while others had no action.

All the active agents, in their nature, are very far removed the one from the other; and there is evidently no link between the physical, chemical and biological agents that are utilized. All act on the bacteria in a different manner but they have, nevertheless, without exception, a common characteristic: they possess some degree of harmfulness for microbes, which may be slight or marked, and it is probably this common property that gives rise to the appearance of L elements.

It is necessary to distinguish between naturally appearing L forms as in the first discoveries of Klieneberger-Nobel, and L forms induced by penicillin (Pierce) and other factors.

The method of normal reproduction of microbes is perfectly understood. In the complete vegetative state they divide by fission.

Aberration from this physiological course is certainly due to an outside agent. In the case of natural L forms, the mode and moment of action of this outside factor are not known. The L elements are presumably pre-existing in the culture, lost amongst the millions of bacterial cells, and only good luck leads to their being observed.

The addition of penicillin to culture media or the action of this or that more or less toxic substance, only check normal bacterial growth, 'liberating' in some way the L forms, and thus making them more apparent to our eyes.

Some investigators think that, according to this hypothesis, there would be no stimulation. Others, on the contrary, suppose that the various agents used since Pierce, while perhaps slightly retarding bacterial development and hindering it, act by means of quite another mechanism altogether. They 'attack' certain ones among their number, and provoke in the sensitive cells a strange defence reaction that may be none other than the appearance of L elements and the starting point of a developmental cycle. Neither of these hypotheses has yet been confirmed by experiment.

Methods of culture

L forms can be grown away from their parent bacteria. Many media have been proposed. All are complex; most are solid or semi-solid media containing nutritive substances in abundance (peptone, serum or blood plasma, ascitic fluid), media which, after seeding, should be maintained in a fairly damp atmosphere. The presence of oxygen is not necessary and some L forms develop anaerobically without there being here, moreover, any connection whatever with the requirements of normal bacteria. Liquid media, such as meat broth enriched with blood serum, ascitic fluid, etc, can also be used. It is possible to seed cultures from solid media in liquid media. Some research workers have grown L forms in chick embryos during incubation.

L form colonies always take a long time to grow. When they have appeared, they can be maintained either in the oven at 37°C, or in the refrigerator.

Colonies of L forms are not visible or are very difficult to see with the naked eye, and can be seen well only under a microscope. They form microcolonies of about 20-500μ in diameter, and rarely, large colonies up to 2 mm in diameter. They are round with an opaque centre, dense, and do not resemble the germs from which they arise.

What are the morphological aspects of the elements constituting these colonies? The problem is an extremely complex one. It must be borne in mind that its solution can be reached only by repeated microscopic observations that are difficult and often erratic; and it must be remembered too that the L forms in no way resemble the bacteria from which they arose, and that all previously acquired knowledge is no longer of use to us. The L elements finally go through a developmental cycle which as a rule should be complete, since, starting from a normal form of bacterium, it should return to it.

Observations show that the reality is more complex than was at first believed. The simple cycle: bacteria—L form—bacteria is not invariably followed. At certain times and under certain conditions L forms reproduce themselves perpetually, as a stable form, and do not show any tendency to return to the normal form of the bacterium from which they arose.

Cycles of development

Figs. 2 and 3 show in detail how the present-day microbiologist regards this development of L forms. Normal microbes subjected to the action of penicillin, for example, are the starting point (fig. 2a).

Some bacteria change in a few minutes into small round bodies of 4-5μ in diameter, made up of a nuclear region and a ring of cytoplasm. These are called 'intermediate forms' (fig. 2b).

The intermediate forms now enlarge up to 15, 25 or sometimes 50μ, becoming the so-called 'globular bodies', 'round bodies', or 'giant forms'. Their internal texture is not clearly defined (figs. 2c and d), but in the centre of most of them there appear granulations, called 'dwarf forms', of very small dimensions, measuring in fact a millionth of a millimetre (125 mμ-200 or 300 mμ). They seem to be rather fragile and possess a very special property, due to their smallness, of being able to go through the walls of filters which retain normal bacteria (fig. 2d).[2]

The giant forms sometimes give rise to small elements having the appearance of very fine bacilli (fig. 2e). The giant forms break up and the elements they contain are liberated (fig. 2f).

Two modes of development have been described for the liberated bodies. In one, the dwarf forms enlarge and return to the intermediate form already described (fig. 2g) and these are then again transformed into giant forms (fig. 2i). Multiple division now leads to the formation of filaments which join together and organize and 'reconstitute' the microbe used at the beginning of the experiment (figs. 2, j towards a).

In other cases, if the medium used continues to be unfavourable to the culture of normal bacteria, for example, if penicillin addition is continued, the dwarf forms begin another cycle from which they will not escape. They return to the intermediate form, then to the giant form (fig. 2, g towards d) and then produce a second generation of dwarf forms which in their turn are freed, giving rise to fresh intermediate forms, and so on (fig. 2, d towards f, then towards g, then towards d, etc). This 'internal cycle' is of considerable biological interest.

The experimenter, by adding substances to a culture, has been able to transform a bacterium of normal dimensions, non-filterable and reproducing by fission, into another form, difficult to see, of much smaller dimensions, filterable, and which reproduces by an altogether different mechanism.

Investigators call the beginning and continuity of this cycle 'fixation of L forms', and apply the term, 'fixed L forms' to those forms which run through it endlessly. Their appearance and fixation can sometimes be long (two years in some experiments) and relatively short in other cases.

L forms can be produced *in vivo*. Numerous experimenters have inoculated normal bac-

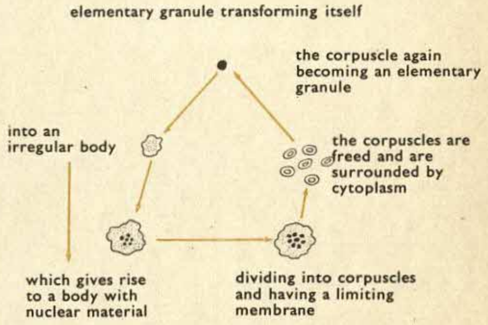
elementary granule transforming itself
the corpuscle again becoming an elementary granule
into an irregular body
the corpuscles are freed and are surrounded by cytoplasm
which gives rise to a body with nuclear material
dividing into corpuscles and having a limiting membrane

Fig. 5 Cycle of development of an organism of pleuro-pneumonia. (After Wilson and Miles, 1955.)

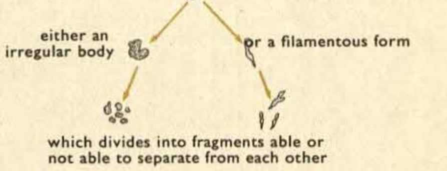
elementary granule transforming itself into
either an irregular body
or a filamentous form
which divides into fragments able or not able to separate from each other

Fig. 6 Possible development of an organism of pleuro-pneumonia of cattle; reproduction by segmentation. (After Wilson and Miles, 1955.)

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(2) *Bacteriologists use filters made of unglazed porcelain, of terracotta and of other substances to separate organisms of different sizes. The pore size of the filter can be varied. Certain filters retain all visible microbes of dimensions between 4 and 6-7μ and can be used for the purification of liquids. These same filters allow very small particles to pass, in particular, dwarf forms of the* L *cycle, which are therefore 'filterable', sharing this property with other submicroscopic particles which we shall refer to.*

(3) *See the chapter 'The Bacteriophages'.*

teria into sensitive animals that have been treated with penicillin and have often recovered L forms from the organs of the animals. L forms have also been recovered from sick persons naturally infected with germs sensitive to penicillin, and they can thus be as easily induced in the organs of man or animals as in culture media.

L forms have also been found in the organs of normal healthy animals that have not been treated in any way. The sera of man, horses, cattle and rabbits have all yielded L forms. The individuals and animals in which these findings were made were normal and showed no sign of disease in the days following the examination.

L *forms and diseases*

Are the L forms pathogenic, that is to say, can they originate disease? Experiments designed to demonstrate a connection between L forms and disease have been very numerous, and in the vast majority of cases have given negative results. But to plead categorically that the L forms can never provoke disease would be a serious error. In face of every problem, working hypotheses must be made. Some of these hypotheses and comparisons of facts—perhaps rewarding—can be filled out.

At the end of the last century (1898) Nocard and Roux cultivated for the first time the microbial agent of a serious disease of cattle, pleuro-pneumonia. In 1923-5, Bridé and Donatien studied contagious milk fever in goats, and for the first time succeeded in cultivating the germ causing the disease. The agents of these two diseases are much smaller than normal bacteria; their morphology is very peculiar and they are non-filterable. Since 1925 numerous researchers, mainly in Britain, have found similar organisms in rats, mice and man, in sewers, soil and dung. They have used symbols, that are now widely accepted, to designate these germs.

The microbial agent of pleuro-pneumonia of cattle, the pleuro-pneumonia organism, is referred to by the letters PPO, and that of goat milk fever by the letters AGO. The microbes of the rat, mouse, etc, the pleuro-pneumonia-like organisms, are represented by the letters PPLO.

The polymorphism of the PPO, AGO and PPLO organisms is extreme. They resemble L forms in many ways, but up to now their identity with L forms has not been confirmed.

Two essential points seem to be agreed, namely that L forms have a developmental cycle starting from an element called a 'minimal reproductive unit', which is filterable and has a diameter of 0·15-0·28μ. The other phases of the cycle are still disputed and seem to vary, as shown in figs. 4, 5 and 6. Some have a complex cycle co-existing with an internal cycle; others have a simpler cycle. Many further observations and great patience will be needed to unravel the details of the cycles.

We have distinguished 'natural' L forms and 'induced' L forms and have emphasized the experimental origin of the latter. However, we now know that there were L forms before the advent of penicillin and that they can be found normally in organs. There is little likelihood that one of the chemical or physical factors mentioned could have originated their appearance.

The 'agent' can only be 'something' very frequently found in organs, water, dung, etc, capable of affecting bacteria and of playing a role similar to that of penicillin.

Bacteriologists know perfectly well that these 'agents' exist. The best known are weak antibiotics and bacteriophages.[3]

Aerobic-Anaerobic: *certain microbes can develop only in the presence of atmospheric oxygen. These are the aerobes (eg diphtheria bacillus). For others oxygen is harmful, and these are the anaerobic microbes (eg tetanus bacillus). There exist all degrees between normal aerobic and strict anaerobic organisms.*

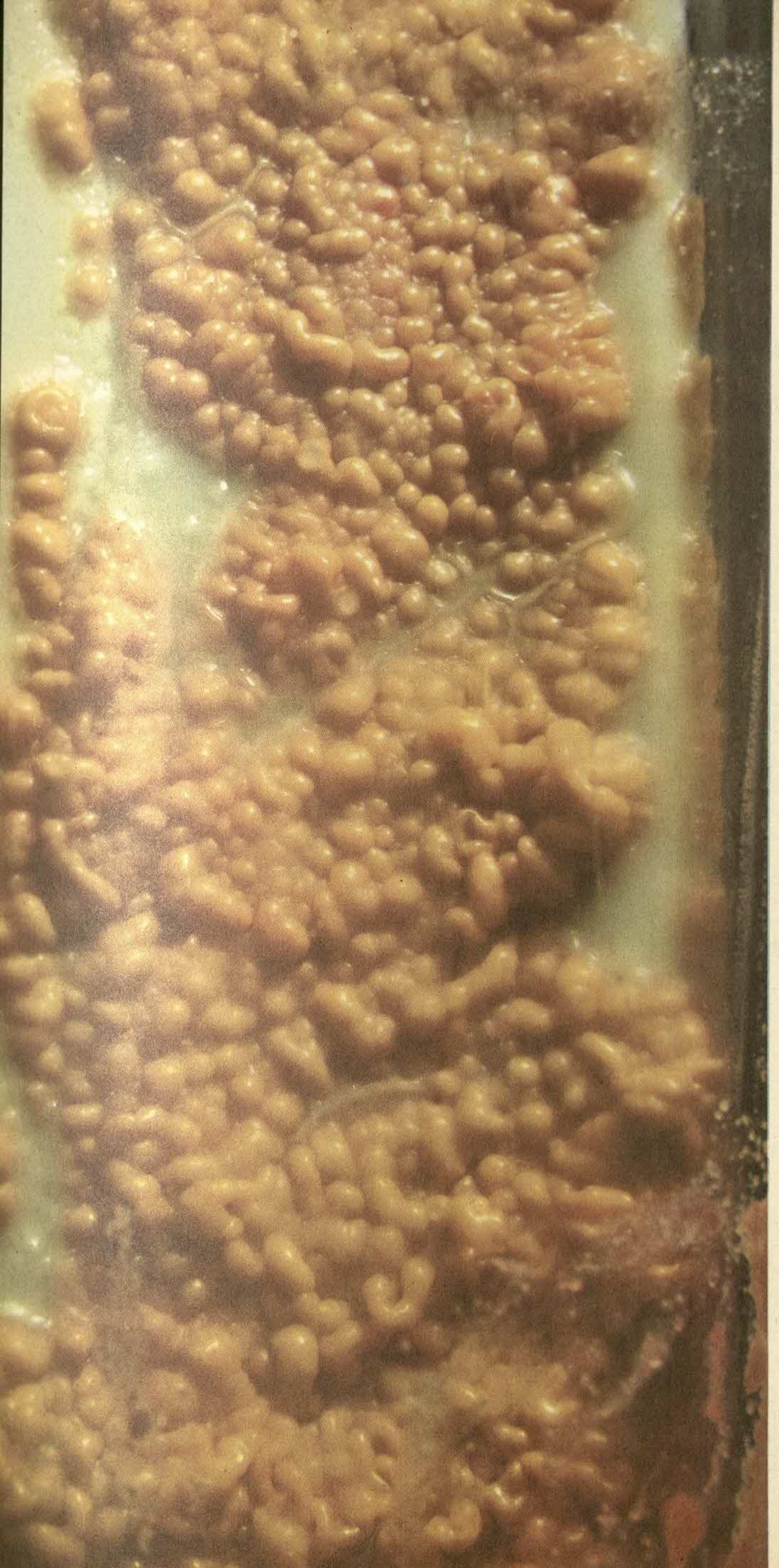
Escherichia coli: *commonly called the colon bacillus or* Bacterium coli. *Very abundant in the intestine in man where it can give rise to urinary infections and sometimes general infections. It is a bacillus 3-4μ long and very mobile.*

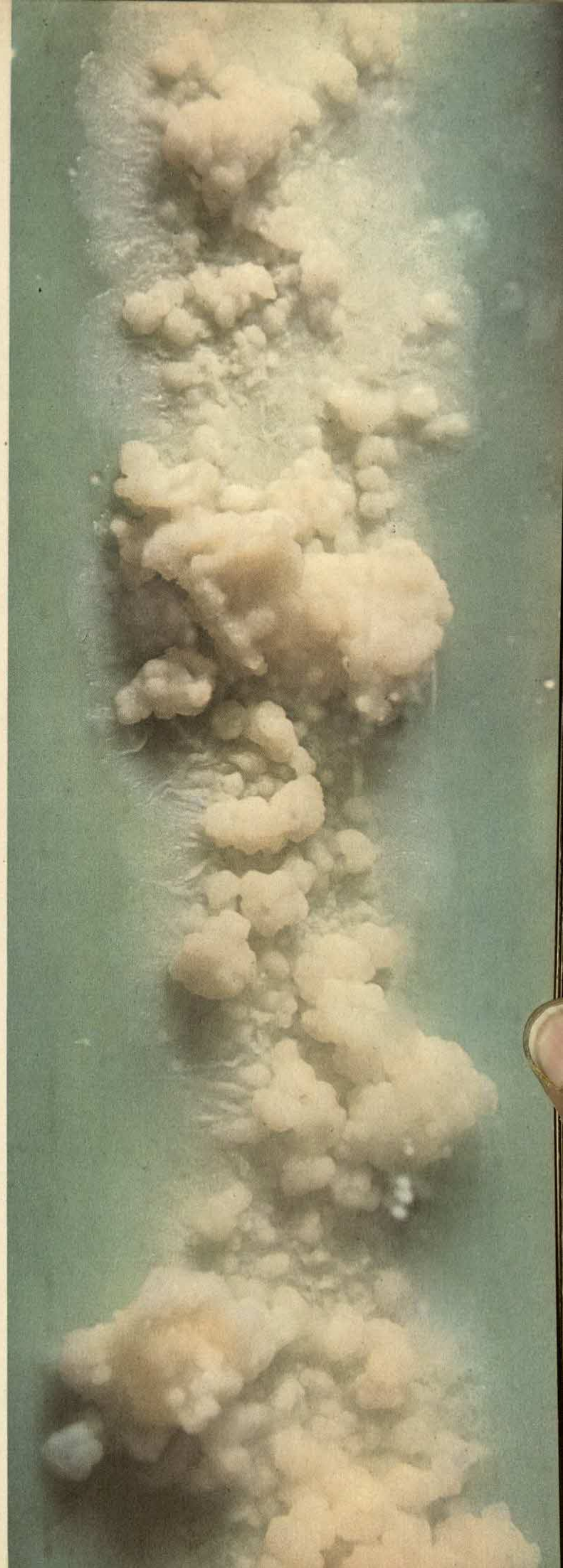
Pleuro-pneumonia of cattle: *pleuro-pneumonia is an infection of cattle characterized by an inflammation of alveolar and bronchial tissue with the formation of sero-fibrinous pleural fluid. This disease is found throughout the world, except in India, North America and Western Europe where sanitary measures have led to its disappearance. The disease is transmitted from a sick animal to a healthy animal by droplets of saliva containing the microbe; mortality is high.*

Streptobacillus moniliformis, *now called* Haverhillia moniliformis. *Non-motile slim rods, 0·1 to 2 or 3μ, that grow at 37°C on media containing blood, ascitic fluid, etc. Their vitality is weak. The agent in man of infectious septicaemia, also affecting the joints, produces in the mouse a fatal disease with swelling of the feet, arthritis and conjunctivitis.*

Spherophorus necrophorus: *a slender bacillus (1μ) of variable length (up to 100μ). The microbial agent of a disease in horses, pigs, cattle (lesions of the hoof, pastern, dermatitis and necrosis of the skin and lips, brain abscess, stomach lesions, etc). Does not seem dangerous to man.*

(Right) After months of culture in an incubator at a suitable temperature, bacterial colonies develop that are visible to the naked eye and contain millions of organisms. The figure shows Mycobacterium phlei *after two months' culture at 37°C on Löwenstein's medium, and the tuberculous bacillus of Calmette and Guérin (B.C.G.) after a month's culture on Löwenstein's medium. (Photo Lod. Experiment carried out by J. G. Marchal).*





The protoplast

Giulio A. MACCACARO

In the present state of our knowledge we can say for certain that a normal bacterial cell comprises a cytoplasm, a nucleus within this cytoplasm, a membrane on the surface of the cytoplasm, and, surrounding the whole, a sheath or cell wall. Recently it has been found possible to keep alive in suitable media bacteria deprived of their cell wall, which has been destroyed by means of an enzyme, or prevented from forming, by means of an antibiotic. Bacteria thus denuded have been given the name of protoplasts. Is this a matter of simple curiosity? By no means; for these abnormal cells are better suited than forms generally observed for carrying-out certain physiological investigations.

Bacteria have been classified as belonging to the world of plants, and various physiological arguments justify this classification, as does also a particularly valid anatomical argument. The animal cell is delimited by a cytoplasmic membrane of which the principal component is protein, but in the bacterial cell, as in the plant cell, a similar membrane is surrounded by a rigid wall which is morphologically well differentiated and is composed mainly of polysaccharides.

It is precisely to the clear chemical and morphological individuality of this cell wall that we owe the recent perfecting of methods allowing the wall to be removed without the internal cellular structures being destroyed in the process. When thus deprived of its essentially plant-like characteristic, the bacterial cell still retains certain of its properties; it nevertheless loses some and acquires others. After this transformation it is known as the protoplast.[1]

The study of the protoplast, of great interest for the understanding not only of bacterial biology, but also of cellular biology in general, now profits by the important progress that has been made in the observation and culture of bacteria.

The techniques of electron microscopy are constantly being improved, enabling almost all phases of bacterial life to be perceived and analyzed. Methods of preparation have also reached a stage of refinement which has made possible the experiments here described.

Origins and methods of preparation of protoplasts

In order to prepare a protoplast, two possibilities, relatively simple—at least as a rule—are open to the research worker: either to remove selectively the wall of normal cells or to prevent the formation of this wall in cells of a new generation.

The action of lysozyme on Gram-positive[2] germs in general has been known for a long time, and in particular on those bacilli belonging to the strains *Sarcina* and *Micrococcus*. Among the latter, the *Micrococcus lysodeicticus* is, as its name implies, very sensitive to the action of this enzyme. The chemical site of the enzymatic attack is known: the lysozyme is a mucopolysaccharidase[3] and the cellular wall of Gram-positive bacteria is composed for the most part of mucopolysaccharides, including sugars, amino sugars, and certain amino acids.

Within recent years (McQuillen, 1955), a new fact has been established. In suitably hypertonic media[4] the cells of *M. lysodeicticus* treated with the lysozyme are not destroyed but are transformed into spheroid bodies which are larger than the original cell and which survive as long as the medium is not diluted. When it is diluted, however, the spherical bodies quickly burst and lysis is completed.

The lesson to be learnt from this experiment is clear: the lysozyme does not affect the

(1) *The term* protoplast *was first used by Mayer (1920) to indicate the whole living cellular matter (nucleus, cytoplasm, membrane, etc) with the exception of structures considered non-vital such as the capsule, insoluble inclusion bodies, and the vacuoles containing the food reserves, etc.*

(2) *Gram staining (1884) consists of treating a preparation with a solution of gentian violet and then with an iodide-iodized solution which acts as a mordant to fix the stain. Decolorization with alcohol is then carried out: certain germs rapidly lose their colour and are called* Gram-negative*; others remain stained and are called* Gram-positive.

(3) *A* mucopolysaccharidase *is an enzyme capable of hydrolyzing mucopolysaccharides, which are large molecules composed of chains of amino sugars. The enzymatic hydrolysis breaks up these chains into their constituent parts.*

(4) *Of two solutions, the one which has a higher osmotic pressure than the other is called* hypertonic. *A solution in which a cell is placed is hypertonic in relation to that cell, if its osmotic pressure is greater than the pressure within the cell.*

(5) *If two solutions of different concentration are separated by a semi-permeable membrane (that is, one which allows the passage of the solvent but not the solute) the passage of the solvent is made from the weak solution to the strong one. Such a passage can be prevented by applying a system of pressure in the opposite direction. This pressure is the measure of that exerted upon the membrane by the difference in the concentrations, and is called* osmotic pressure.

(6) *The* lipids *or fats are organic substances composed principally of carbon, hydrogen and oxygen, differently soluble in organic solvents (alcohol, ether, acetone, benzene, etc) but only slightly soluble in water.*

(7) *An organic substance is of the* chelating *type when it is*

Continued overleaf

deeper structures of the cell but only its wall, and lysis results from intra-cellular pressure. Because of this pressure the cytoplasmic membrane gives way as soon as it is no longer supported either by the wall or by an equivalent osmotic pressure[5] of the medium in which it is suspended. This phenomenon was described by Tomcsik in 1951.

Thus is created the 'protoplast'—the bacterial cell without a wall—as Weibull (1953), who obtained analogous results with *Bacillus megatherium*, has named it.

Up to now we have been considering the Gram-positive bacteria and, in fact, a method such as that just described would be quite inadequate for preparing protoplasts from Gram-negative bacteria. In the latter the membrane is much richer in lipids[6] but poorer in mucopolysaccharides, and it also possesses a series of amino acids (aromatic acids, sulphurated acids, arginine, proline) which are absent from the walls of Gram-positive cells.

Thus, not only is the quantitative value of structure substrate for lysozyme in the structure of the wall of Gram-negative germs less, but very probably the substrate itself is somehow protected by components of different nature. It is thus not surprising to find that the same enzymatic treatment, which in protoplasts of the cells of *Micrococcus* and *Bacillus* gives rise to a transformation, has no effect whatever upon the cells of *Escherichia* and *Pseudomonas*, to take two examples. The effect can be reproduced only by working in a strongly alkaline medium (Zinder and Arndt, 1956), or by adding a chelating agent[7] such as ethylenediaminotetracetic acid (Repaske, 1956). However, chemical, immunological and biological tests have shown that the spheroid bodies obtained in this way retain more than a trace of the cellular wall.

There is general agreement with regard to describing these formations as 'spheroplasts', whilst reserving the name of protoplasts for those in which the total disappearance of the cell wall can be confirmed by all the available means in our possession.

But, as mentioned above, the problem had already been tackled at about the same time in a quite different way: by preventing the synthesis of the matter which goes to make up the cell wall, without affecting any other cellular matter. The first success in this was achieved by Liebermeister and Kellenberger in 1956, and by Lederberg in the same year. These researchers showed that, by exposing growing cultures of *Proteus* and of *Escherichia* to the action of penicillin in adequate hypertonic media, they could obtain the transformation of cells into spherical bodies which were osmotically fragile; in other words into protoplasts or at least into spheroplasts. Other studies have followed (Park, 1958) and led to an interesting conclusion: such an effect would be the manifestation of a disturbance caused by the antibiotic in the synthesis of a uridinic nucleotide,[8] which in its turn is the biochemical precursor of the matter of which the cell wall is composed. If things stop at this point, penicillin does not give rise directly to bacterial lysis; but it prevents the organism from forming the structure which will screen it from the effects of the releases due to the difference in intra- and extra-cellular osmotic pressures.

In an even more refined experiment, McQuillen (1957) obtained spheroplasts of *Escherichia coli* by growing a strain incapable of synthesizing diaminopimelic acid[9] in a hypertonic medium deprived of this substance. Cells of this strain, being incapable of synthesizing and not finding it in the medium, grow without a wall or with a mere skeleton of a wall: therefore they show their spherical form, their osmotic weakness and, in short, their character as 'spheroplasts'.

Form and composition

The protoplast is bound to be spherical. The osmotic pressure of several atmospheres in its interior would make it explode if this were not counterbalanced by the equal osmotic pressure of the medium in which it is placed. Because of this and owing to simple and well-known laws of physics, it tends to take a shape that reduces its surface in relation to its volume, or in other words that is to say, a spherical shape. The progressive appearance of this sphere from the bacterial body and the transformation of the latter have been represented and illustrated by microphotographs obtained by recent techniques.

One frequently observes vacuoles in the interior of the protoplasts which probably bear witness to a lack of homogeneity of the cytoplasm. One can sometimes also recognize two zones, distinguished from each other by the difference in their density, which give the body a curious lunar aspect (fig. 1); this phenomenon is perhaps due to a residual cell wall which continues to adhere to one part of the spheroplast.

The protoplast of a cell provided with flagella[10] retains these but loses the power of locomotion (Weibull, 1953): this disappears the moment the cell wall ceases to be rigid, as

Continued from previous page

capable of retaining multivalent metallic ions (calcium, magnesium, etc) by two lateral chains.

(8) A nucleotide *is composed of a molecule of phosphoric acid joined to a molecule of ribose, which in its turn is combined with a purine or pyrimidine base. A long chain of nucleotides of this type makes up ribonucleic acid* (RNA). *If in place of the ribose there is deoxyribose, the acid is called deoxyribonucleic acid* (DNA). DNA *is the constituent of chromosome material and therefore the carrier of genetic information.*

(9) Diaminopimelic acid *is a specific and essential constituent of the cellular wall of all bacterial species (except Gram+ cocci).*

(10) Flagella *are thread-like appendages, the number of which can vary from one to a few dozens per cell. By their movements the bacterial cell can move in the medium in which it is suspended.* Flagella *are composed of fibro-elastic protein.*

(11) Fimbriae *are thread-like appendages distinguishable from the cilia by being shorter, finer, more numerous and more rigid. Since they have no connection with the mobility of the cell, it has been suggested that they have adhesive properties.*

(12) Cytochromes *are pigments, containing iron, that are essential for cellular respiration.*

(13) *A bacterial cell is endowed with an extraordinary enzymatic make-up. The* enzymes *are divided into two large groups: those which are always present in the cell and are therefore called constitutive, and those described as adaptive, which are produced only in response to contact with a corresponding substrate.*

(14) *See the chapter 'The Sexuality of Bacteria' by G. Maccacaro.*

(15) Transformation. *This term indicates the possibility of transferring characters from one cell to another by simply placing the second cell in contact with a* DNA *extract of the first. The*

Continued on next page

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transformation thus obtained is irreversible and hereditary.

(16) *A bacterial medium is called 'minimal' for a given species when it contains only the simple essential elements for multiplication. For* E. coli, *for example, a* minimal medium *is composed of a solution containing ammonium salts as a source of nitrogen and glucose as a source of carbon and energy.*

HISTORICAL NOTES

1900

A. FISCHER described the phenomenon of 'plasmoptysis' in which, in a hypertonic medium, the wall of the bacterial cell breaks locally and the cytoplasm escapes as a bubble which remains united to the cell or which

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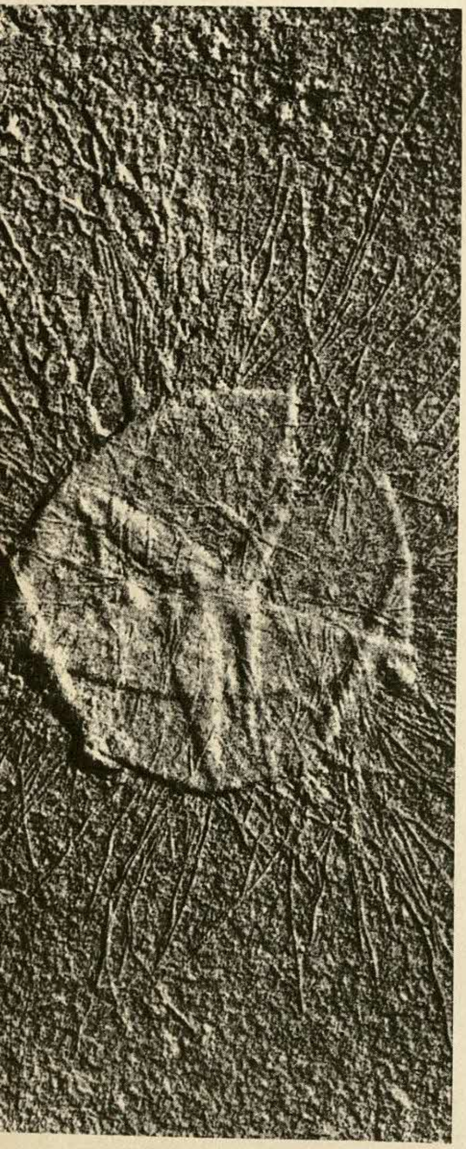

Fig. 1. The protoplast is a bacterial cell deprived of its wall. The obtaining of such cells is no mere scientific diversion: it clarifies the mechanism of action of certain antibiotics. Moreover, any substance capable of inhibiting the synthesis of the constituents of the cell wall would be bound to lead to the death of the cell.

though its rigidity furnished the support necessary to the movements of propulsion by the flagella (Stocker, 1956). The spheroplast of *E. coli* also protects those other tiny and numerous filamentary appendages called fimbriae[11] (Maccacaro, 1958).

Chemical analysis reveals in the protoplast all the constituents of the intact bacterial cell from lipids to proteins, and from cytochromes[12] to nucleic acids. Certain substances, however, such as diaminopimelic acid, are lacking. A more convincing proof of the specificity of the structure of the cellular wall would be difficult to find.

The physiology of the protoplast

Deprived of its natural protective envelope, modified in form and volume, impaired in its fundamental physical and chemical properties, the bacterial cell transformed into a protoplast nevertheless retains almost all its physiological properties. This applies not only to endogenous respiration and the oxidation of certain fundamental substrates (Weibull, 1953), but also to the synthesis and the activity of more specialized enzymes.[13]

When protoplasts are placed in contact with various substances marked by the introduction of radioactive atoms into their molecule, the rapidity with which these substances are absorbed and transformed by the protoplast indicates the continuing presence and activity of its principal constituent enzymes (McQuillen, 1955; Hunter and colleagues, 1957). It has been seen that in the same way a protoplast put in contact with various substrates produces a notable quantity of corresponding adaptive enzymes (McQuillen, 1956). Even protoplasts burst by osmotic pressure and emptied of a large part of their contents retain a certain number of enzymatic activities, and this entitles us to draw a conclusion of extraordinary interest for general biology: the site of these activities is furnished in large measure by the cytoplasmic membrane itself (Spiegelman and colleagues, 1957, Butler and colleagues, 1958).

When one considers, on the other hand, that the protoplast retains its ability to transform itself into a spore (Salton, 1955), two important questions present themselves. First, is it capable of growth and multiplication? Second, is it capable under suitable conditions of returning to the normal bacterial form?

The answer to both these questions is unfortunately still in doubt. In fact, by maintaining a suspension of protoplasts in a suitable culture medium and under favourable conditions of incubation, it is possible, with time, to measure a linear increase of their global mass, but not a numerical increase (FitzJames, 1958). It is as if the synthesis of protoplasm could be continued without, however, leading to the formation of a new cell.

On the other hand, no clear proof exists of the return of the protoplast to the initial bacterial form; when it has been observed, we cannot be quite sure that this is not due to elements having conserved some remains of cell wall. Reversion has been described, in fact, as being frequent in spheroplasts due to penicillin (Lederberg, 1956).

Genetic possibilities

The last fifteen years have seen the development of a new discipline of extraordinary interest: the genetics of micro-organisms.[14] An important date in its brief history was the day in 1946 when an American student, Joshua Lederberg, was able to demonstrate that two bacterial cells can conjugate and exchange a part of their genetic information.

Later (1953), through the researches of Lederberg, of the Italian, Cavalli and of the Englishman, Hayes, it was discovered that one of the two cells had male properties (donor of genes) and the other female (recipient of genes); and also that cells of the first type (known as F^+) could transmit their sexual type to those of the second (known as F^-) by simple contact.

It was also Lederberg, in collaboration with Saint-Clair (1958), who demonstrated that the spheroplasts of *E. coli* produced by penicillin are capable of conjugating in the same way as intact cells; and Hagiwara (1958) has confirmed that transmission of the sexual type takes place also in spheroplasts.

As in the meantime it had been shown (Maccacaro, 1955) that bacterial sexual typology manifests itself at the level of the cellular surface, and that a significant portion of the cell wall remains, as we have seen, on the spheroplasts, it is not improbable that the latter owe the retention of their genetic properties to these cell-wall remains.

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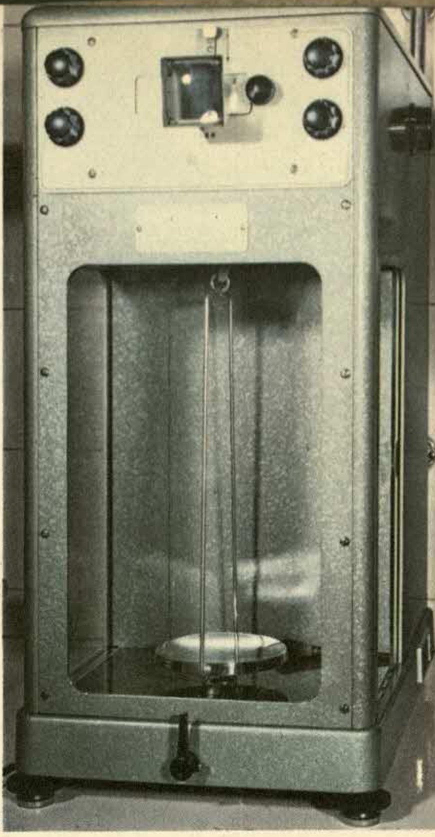
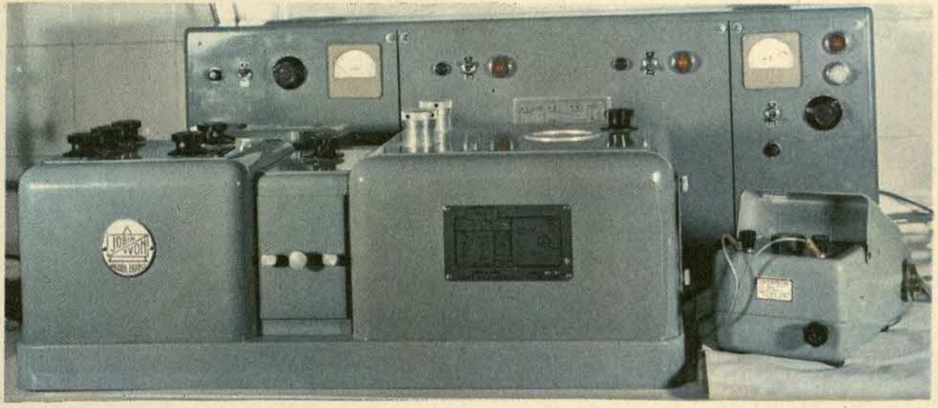


4

Laboratory equipment

Inside the laboratory: (1) micromanipulation, owing to highly refined pneumatic control, allows bacteria to be selected one by one; (2) Warburg apparatus, used in the measuring of gaseous exchanges of tissues or of cells (the tubes contain samples for analysis); (3) a spectrophotometer; (4) precision balance capable of weighing below one milligram; (5) a corner of the laboratory: note the usual apparatus. A technician is about to inoculate a microbial culture into a rabbit. (*Photo Lod. Pasteur Institute*).

3



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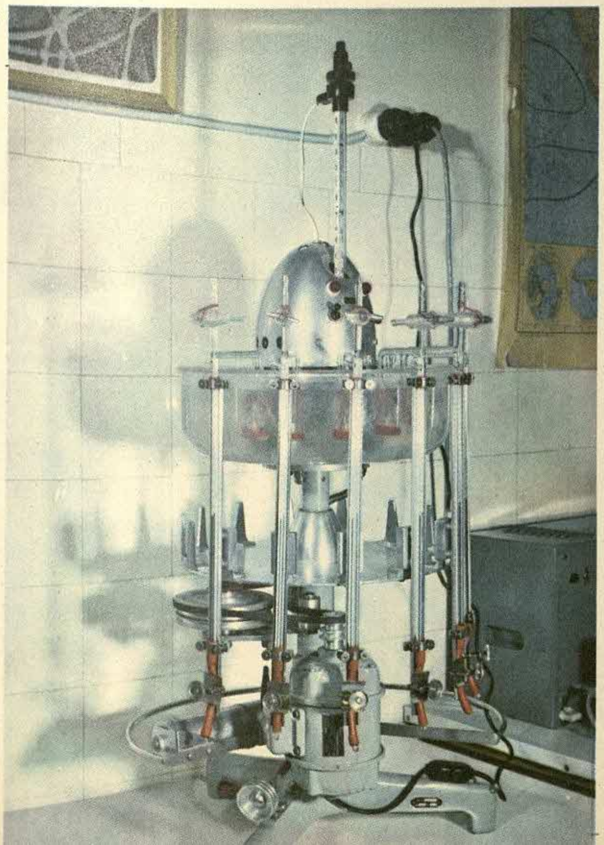

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is detached as a small spheroidal mass. The opposite phenomenon called 'plasmolysis' is seen when the cell finds itself in a strongly hypertonic medium. Its cytoplasm retracts and with it the cytoplasmic membrane which is thus detached from the cell wall.

1922
A. FLEMING found in secretions and animal tissues an enzyme, which he called lysozyme, capable of producing lysis of bacterial cells.

1952
J. TOMCSIK and J.-B. GUEX-HOLZER, while studying the lysis of Bacillus megatherium *by lysozyme, noticed that in saline solution the treated cells became rounder, liberating small spherical masses of cytoplasm.*

1953
C. WEIBULL made independent observations analogous to those of TOMCSIK and GUEX-HOLZER and demonstrated that the spherical masses were cellular bodies denuded of their wall and delimited by cytoplasmic membrane. He named them protoplasts.

1956
K. LIEBERMEISTER and E. KELLENBERGER and, independently, J. LEDERBERGER, discovered that in a hypertonic medium Gram-negative bacteria sensitive to penicillin were transformed into protoplasts under the influence of the antibiotic. This latter gives rise in the cell to a metabolic disturbance which prevents the synthesis of substances essential for the formation of the cellular wall.

1957
K. McQUILLEN obtained protoplasts of a strain of Escherichia coli, *incapable of synthesizing diaminopimelic acid and which developed in a medium deprived of this substance. Diaminopimelic acid is a specific and essential constituent of the cellular wall of several bacterial species. Cells which are incapable of synthesizing it and which do not have it in their nutritive medium, grow without a wall, ie, as protoplasts.*

Another remarkable chapter of bacterial genetics is that relating to transformation.[15] But, so far, it has been possible to observe this only in a very small number of species, which for the most part are Gram-positive. This is probably due to the wide diffusion of enzymes capable of destroying DNA before it can be integrated into the receptor cell, or perhaps to the difficulties experienced by such a large molecule in passing through the cellular wall of many species, for example *E. coli.*

Chargaff and his colleagues (1957) considered that perhaps the removal of the cell wall makes possible the entrance of the transforming principle in the species where the phenomenon appeared incapable of proof: in other words, the protoplast would have been able to be transformed at a time when the corresponding intact cell was not. These researchers prepared spheroplasts (using penicillin) from a strain of *E. coli* incapable of growth in a minimal medium.[16] They then placed them in contact with DNA extracted from cells of the same species, but belonging to a strain capable of growth. After they had been washed, the spheroplasts were placed in the medium where previously they had been unable to grow, and it was found that a certain number of cells multiplied and produced colonies.

If these experiments can be extended and confirmed, another large step will have been made towards the understanding of the mechanisms of exchange of genetic information between different cells.

The protoplast and bacteriophages

The bacterial cell, like the animal or plant cell, is sensitive to the action of the specific viruses called bacteriophages, which infect them and lyse them. But a bacteriophage cannot infect a bacterial cell unless it finds on the surface specific receptors to which it can attach itelf. Since these receptors belong to the cell wall, it might be expected that when a bacterial cell is deprived of its wall it would cease to be capable of infection by the virus to which it is sensitive. Experiments confirm this so clearly, that we now consider the fact of being still able to adsorb a few bacteriophages as proof of an incomplete destruction of the cell wall (Weidel and Primosigh, 1958).

If, however, the cell has been previously infected by the phage and is afterwards transformed into a protoplast, the multiplication of the virus still takes place as usual (Brenner and Stent, 1955). This confirms once again that the protoplast retains undamaged a large part of the physiological properties and biochemical potentialities of the intact cell.

Another interesting fact is that, under certain conditions, bacteriophages injured by thermal and osmotic treatments are capable of infecting spheroplasts which cannot be infected by the intact phage (Spizizen, 1957). Perhaps the physical damage to the virus liberates some active components which are capable of penetrating into the bacterial cell by a route other than that of the receptors in the cell wall. However, as the reader will already have gathered, the researches described in these last paragraphs involve a measure of doubt and provisional statement; we have, in fact, reached problems that are being considered in experiments actually in progress and must therefore await further results.

It would be wise, perhaps, to consider the whole of the present scene in perspective.

It may seem to the uninitiated that the study of procedures which lead to removing the bacterial cell from its envelope is little more than a refined diversion on the part of disinterested biologists. This is perhaps true in so far as each research is satisfying to the research worker; but it is also true that these researches have added considerably to the progress of knowledge and always lead to results more general than the questions that suggested them. The study of techniques for obtaining protoplasts has clarified the mechanism of action of certain antibiotics and suggested a possible strategy for research and the use of other chemotherapeutic agents: thus, since it has been proved that the cellular wall has its own special constituents and that the cell without a wall is destroyed by its own internal pressure, it is clear that any remedy capable of specifically inhibiting the synthesis of those constituents is bound to lead to the death of the cell.

We can regard bacteria as model experimental material for the study of cellular biology, and a large part of the knowledge that has been acquired relating to their mode of life, their reproduction and their death has clarified for us certain properties of animal and human cells which are less accessible to experimentation. The study of bacteria deprived of their vegetable envelope has made and will continue to make an important contribution in this field.

Identification of a bacterium

Romolo DEOTTO

Millions of species of microbes exist, many of which resemble one another. How can one separate and distinguish them from each other? At first sight the task seems overwhelming; but, in fact, in his daily practice the bacteriologist succeeds quite easily, because over the years research methods have been perfected which greatly help him. Some of these methods depend upon morphological data and others on biochemical, serological or biological tests.

To identify a bacterium it is necessary to recognize in it a series of characteristics which determine the place it must occupy in the classification of living beings. As all bacteria belong to the class of Schizomycetes, which itself is subdivided into orders, families, genera and species, it is simply a question of determining to which species the bacterium that is being examined belongs. In some cases it may be necessary to identify not only the species but also its variety and type within the species. This will mean determining further characteristics which vary from one strain to another.

Such is the problem which confronts the microbiologist when identification is the object of his researches, or when it serves merely as a premise to the study of the enzymatic properties of an unknown strain, its antigenic constitution, and so on. But how does he proceed when he is at grips with biological entities of which the morphology furnishes so few indications?

By and large, the course he follows in identifying a bacterium is guided by the same criteria which have led to bacterial classification. To classify (and recognize) the microorganisms, one can refer to the data concerning their morphology, culture, biochemistry, serology, and biology.

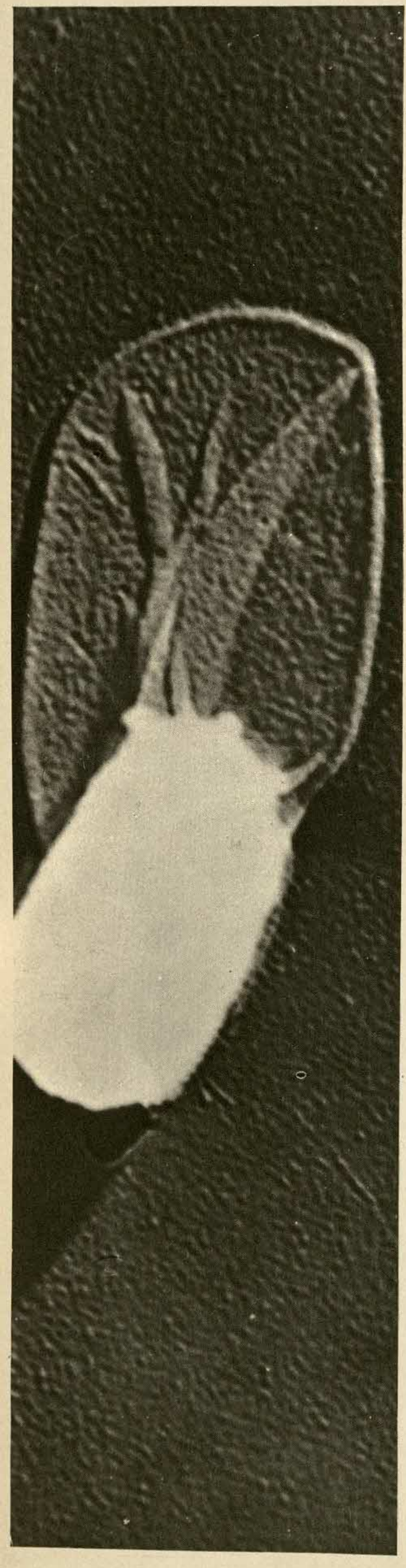
Morphological data

Here the indispensable instrument is the optical microscope. In certain cases it may be useful for observing the bacteria taken straight from a living lesion which presents the clinical characteristics of a primary sore of syphilis. If from a lesion one can remove, by means of a light scraping or the simple application of a glass slide, a small quantity of pathological fluid for microscopical examination, this will show refracting motile filaments in the form of a spiral (like the thread of a screw), and these will be immediately identified by the trained bacteriologist as *Treponema pallidum*, the agent of syphilis. But in this case, in fact, it is not simple microscopic observation which has led to the identification of the germ: the observer has drawn, if unconsciously, upon other biological data founded upon clinical experience, and which, in this particular case, allow him to suppose that the lesion from which material was removed was of a syphilitic nature and that, as a result, the spirochaete observed was very probably *Treponema pallidum*.

In the majority of cases the data provided by direct microscopic examination of fresh material are insufficient for the identification of a bacterium. Observation must be carried out on a specimen that has first been suitably fixed and stained on a glass slide. A very thin layer of the material to be examined (bacterial suspension or pathological material) is placed on a slide and fixed by flaming. The staining solution is then placed on the slide, using the appropriate technique. Some seconds or minutes later, according to the technique adopted, the slide is washed with water and the preparation is dried. The bacterial cells, having absorbed the colour, become much easier to observe under the microscope. These staining techniques furnish the first effectual aid in the identification of bacteria.

(1) Cocci: *from the Greek 'kokkos'. Bacterial cells of round shape, the cocci may occur as isolated cells; or joined in groups of two (diplococci) or four (tetrads); or in groups of eight, looking rather like two tetrads superimposed (sarcinae); or in chains of varying length (streptococci) or in irregular grape-like clusters (staphylococci).*

(2) Lipids: *substances characterized by their molecular content of fatty acid radicals.*



Thanks to the electron microscope, the characteristics of bacteria appear with striking clarity. This electron micro-photograph shows the final phase of sporulation with the collapsed cellular wall empty of cytoplasm, rather like a transparent envelope. (Mag. × 42,000. Istituto Superiore di Sanità, Rome).

There is a method of staining (for example, diluted fuchsin, or methylene blue) which is common for all bacteria. This reveals whether the organism is a coccus[1] (or a diplococcus, a coccus in tetrads, a sarcina, a streptococcus or a staphylococcus), or a bacillus, that is to say, a rod-shaped germ, which may occur in chain form or as an elongated spiral filament.

To make the cilia, the capsule, or the spores appear, special stains are necessary. Each of them provides fresh information and aids identification.

The original method of Gram (1884) with its numerous variants consists, first of all, in staining bacteria which have been fixed on a slide, with a solution of gentian violet. This slide is then treated with an iodo-iodized solution, followed by alcohol, which more or less decolorizes it. Then a second stain differing in colour from gentian violet and called a counterstain, is added. This counterstain is usually fuchsin or safranin.

Under the microscope, such a preparation (provided, of course, that it is in the presence of a mixed bacterial population) will show that certain cells have kept their initial stain; that is to say, the alcohol has decolorized them only slightly, whereas others, decolorized by the alcohol, have taken the colour of the counterstain. The first are called Gram-positive and the second Gram-negative.

Another special method of staining is that of Ziehl. The majority of bacterial species take on colour well, swiftly, and in the cold state, by means of the usual methods. Some species, on the other hand, take on the colourings only in the warm state and in the presence of certain mordants. However, once stained, such bacteria cannot be decolorized, unlike the others, when treated with strong acids. The Ziehl method (also called the Ziehl-Neelsen method) consists actually of staining the preparation with phenolic fuchsin, when in the warm state, the phenol acting as a mordant. Sulphuric or nitric acid is used to decolorize, and safranin, besurine or methylene blue as counterstain. The bacteria which have resisted decolorization by acid are stained red with fuchsin and these are called acid-fast. The others decolorized by the acid and which have taken the counterstain are called acid-sensitive or non-acid-fast. If, after acid decolorizing and counterstaining, alcohol is used as a further decolouring agent, one can still separate the bacteria into those which resist acid and those which resist acid and alcohol.

The Ziehl method divides the bacterial world into two very distinct categories. We know, for example, that the acid-fast germs are much richer in lipids[2] than the non-acid-fast. Besides being useful for the classification and identification of bacteria in general, this method has been particularly valuable for the identification of the tubercular bacillus. If, on staining sputum by the Ziehl method, acid-fast bacilli are observed, and if the clinical data are conformable, one can be certain that the correct diagnosis is that of infectious tuberculosis.

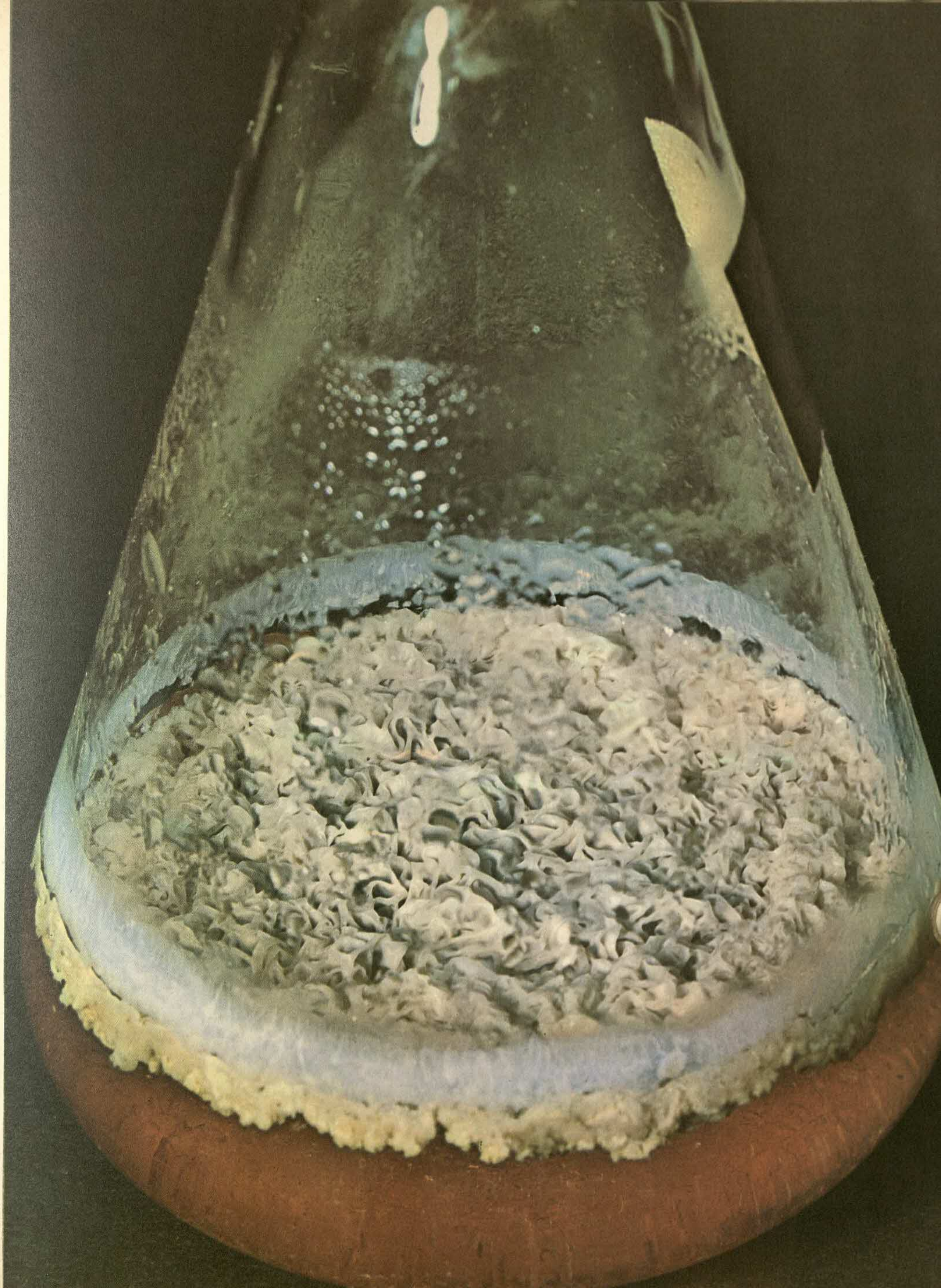
By revealing bacterial shape, and the presence of cilia or of spores, and by furnishing information about the mode of reaction to stains, microscopic observation can provide basic morphological indications for the identification of bacteria. None of these criteria is sufficient by itself; nevertheless, all of them are useful and can lead to the identification of a bacterium.

Data relating to culture

On this subject the reader should also refer to the chapters which follow. The practice of bacterial culture allows us to divide bacteria into the aerobic, the facultative anaerobic, the microaerophilic and the strictly anaerobic.[3] The bacterial world can thus be divided into four sectors: the fact that one bacterium develops better on one medium than on another, is in itself a useful indication. Then again, each bacterial species can, on an adequate medium, present certain characteristics and be seen to behave in a particular manner, thus providing important information.

In a liquid culture medium, some bacteria multiply only on the surface, forming a skin with a characteristic appearance, and leave the adjacent liquid clear. Other bacteria, on the other hand, grow within the medium either in a uniform manner, or forming small aggregates which rapidly fall to the bottom of the tube or flask.

However, it is in cultures on solid agar media that the indicative characters appear most clearly and are most often used. On solid media, bacterial colonies arise by successive



A culture of Mycobacterium friedmanii *on a synthetic medium. (Photo Lod. From the laboratory of J. G. Marchal).*

multiplication of a single bacterial cell. One cell divides into two, then into four, then into eight, and so on (theoretically, at least) until there is a colony composed of a billion or more bacterial cells; a colony which is visible to the naked eye and is perhaps several millimetres in diameter.

Such colonies can be thin or transparent, thick, clear or opaque; they can have a moist, mucous, or chalky appearance; they can be flat, convex, or umbilicate; they can present clear edges, blurred edges, or scalloped edges; they can be whitish, or they can show variegated pigmentations. Each of these characters is, in general, constant in one bacterial species and helps in its identification. Often the characters allow the identification to proceed by elimination: a coloured colony is clearly not of a bacterial species which is known to produce whitish colonies. When one finds a colony which is flat and dry, one knows, similarly that it is not of a species characterized by small, convex, moist colonies, and so on.

Biochemical data

The metabolism of a bacterium determines the changes in the medium in which it develops; its ability or inability to use a predetermined substrate and the diversity of methods of attack on the substrate, are as many expressions of enzymatic activity characteristic of each species of bacterium.

Carbohydrates are nearly always used as a source of energy for bacteria in culture, but different species use different sugars, and have varying methods of attack, forming in consequence different breakdown products.

Take the case of glucose—it can be decomposed to form acids, and these are easy to detect. If a suitable indicator[4] (usually litmus), is added to the culture medium, the change of colour after acid is produced is readily observed.

But the production of acid can be hidden by the ammonia formed as a result of the simultaneous breaking-down of amino-acids[5] by the bacteria.

In this case, the indicator that has been added to the culture medium is of no value. Only the final titration of the sugar would tell us whether or not it had been broken down.

This electron micro-photograph shows a bacillus of Pletridium tetani. *The presence of a terminal spore of a diameter slightly greater than that of the bacillus gives the characteristic drumstick appearance. (Mag. × 19,000. Istituto Superiore di Sanità, Rome).*

Glucose can also be decomposed, giving rise to acids and bases (carbon dioxide). One can demonstrate the existence of the latter by putting into the tube which contains the liquid medium culture (or broth) a much smaller inverted tube in which the gas will accumulate, as in the bell of a gas-jar. If, on the other hand, the bacterium is inoculated at the bottom of a cube of agar, the gel is broken up by the pressure of the gas that has been formed within it.

The breakdown of glucose can extend to the formation of important quantities of short-chain acids such as formic acid, acetic acid, etc. These acids are strongly dissociated and can alter the reaction of a culture medium which was originally buffered, from neutrality to an acidity corresponding to a pH[6] less than 4·5. If some drops of methyl red are then added to the culture broth, it will change colour until it is completely red; this process is referred to as a positive reaction to methyl red. In the opposite case, the colour would be yellow and the reaction would be referred to as negative.

Other species of bacteria break down glucose into acetylmethylcarbinol. This process can be shown by making the medium strongly alkaline with potassium. The positive reaction for acetylmethylcarbinol is a fluorescent red coloration (Voges-Proskauer reaction).

It can be seen therefore that various possibilities are offered as a result of a simple attack on glucose, but one would be able to say as much, with a few variable exceptions, for each carbohydrate.

Certain bacteria are able to use citrates as a unique source of carbon; others are incapable of doing this. Certain bacteria can attack tartrates of which they can distinguish the optical isomers.

Some bacterial species can break down proteins (the proteolytic bacteria). This activity can be shown in many ways. In cultivating bacteria on gelatine, one can not only tell whether they are proteolytic (they liquefy the gelatine) but can draw useful indications from the manner by which the gelatine is attacked. Blood serum, white of egg and fibrin are used in culture media to differentiate the proteolytic activity characteristic of different bacterial species.

The case of milk is particularly interesting. It can be curdled by simple acidification, owing to the breakdown of lactose and the consequent formation of acids. If, in such a case, a suitable indicator is added, it will show an acid reaction. It can also be curdled by an enzyme produced by bacteria, such as rennet. This enzyme curdles milk without attacking lactose and in this case an indicator would not show an acid reaction. If, in addition, the bacteria also possess proteolytic activity, the curds of casein would be secondarily digested, that is to say, liquefied.

Certain bacteria, such as *Proteus vulgaris*, possess the enzyme called urease, capable of splitting urea into ammonium carbonate. Others, while attacking an amino-acid, tryptophane, give rise to indol. Yet others reduce nitrates into nitrites. The cholera vibrio possesses both the two last-named properties. A simple reaction called the cholera red test is used to demonstrate both these properties. This is done by acidifying the culture with sulphuric acid, which forms red-coloured nitroso-indol.

We have referred to the formation of carbon dioxide from sugar metabolism, but bacteria can form other gases—hydrogen, hydrogen sulphide, methane and ammonia. Another bacterial property, which is the expression of biochemical activity but is often considered as a property of the culture, is haemolysis. Certain bacterial species grown in solid media containing blood can lyse the red cells. This is shown by the formation around the bacterial colony of a clear halo very distinct from the red and opaque surrounding mass.

It sometimes happens that this halo takes on a greenish tinge. This constitutes a distinguishing characteristic of certain streptococci. Also, the haemolytic activity appears to be variable according to the type of red blood corpuscles used. Certain bacterial species lyse only the red cells of the horse, others those of the rabbit, and yet others those of sheep or of man. This list of biochemical tests could be much lengthened.

For reasons of economy in time and material, the bacteriologist does not proceed each time to an analysis of all the properties of the unknown bacterium. He restricts himself to the use of a series of special culture media which, thanks to the addition of this or that substance or indicator, allows him to choose, the quickest method of making an accurate identification.

Bacterial species are counted by tens of thousands. The data relating to their morphology, culture and biochemistry, at present catalogued, are about thirty (remembering the long series of sugars). Each character considered divides the field of research into two parts at least; with thirty criteria, one arrives at a theoretical possibility of information of 2^{30}, and this reduces by a factor of a million the initial field of investigation. It can be argued that this procedure is not really so precise because each character does not divide the field of investigation into two equal parts; and several characters are often found together in a single strain. It can also be said that all the catalogued characters give rise to an excessive number of permutations. In practice, the experienced worker uses a much smaller number of tests to identify the strain.

Serological data

A bacterial cell is a mosaic of antigens[7] and when it is inoculated into an animal, it initiates the formation of all the corresponding antibodies. In consequence, a bacterial strain can be identified by testing it against known immune-sera.[8]

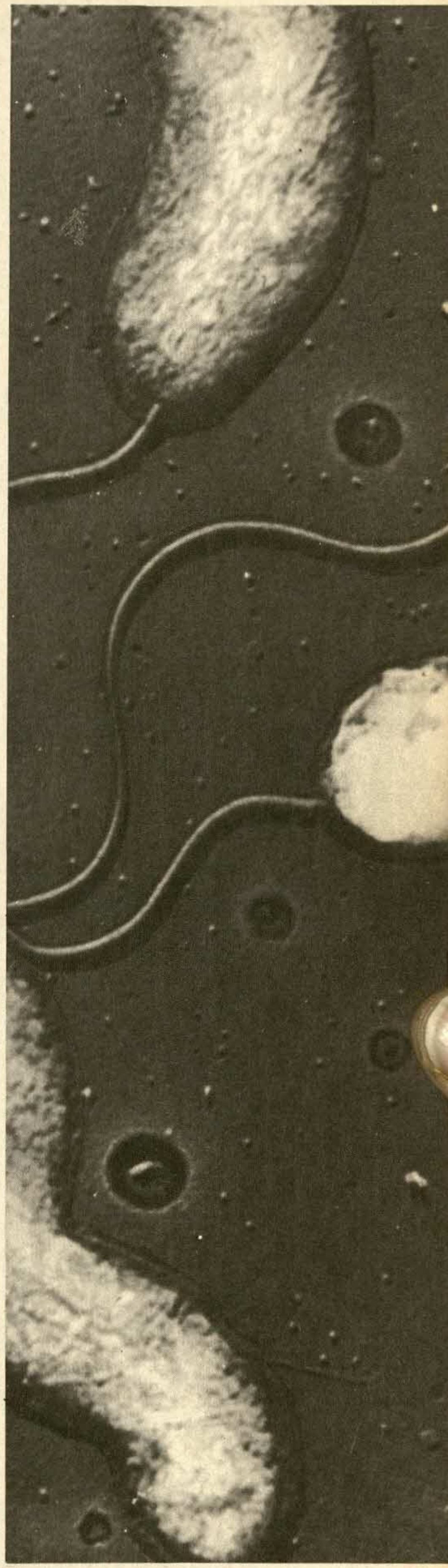
If a strain reacts by agglutination[9] with an anti-typhoid serum, one cannot conclude immediately that it must be *Salmonella typhi*; other bacterial species react in the same fashion. This is due to the fact that bacterial 'groups' can be recognized by the possession of common antigens, in much the same way that different buildings may have in common architectural motifs.

But there are various other criteria and research techniques which allow us finally to distinguish between the specific reaction of the group and the reaction characteristic of the species (taking into account the serological reactions). Moreover, within a bacterial species there are different types which can be distinguished by the presence of antigenic characters (type antigens); and serological reactions allow the different types within bacterial species to be identified with great precision. It is thus that seventy-five different types can be distinguished in the species *Diplococcus pneumoniae.*

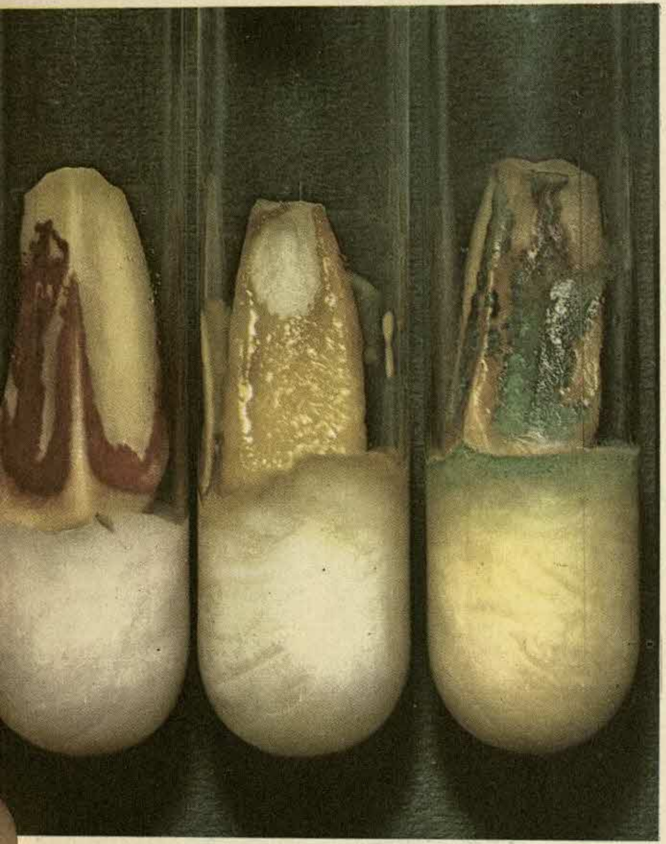
Biological data

There are, broadly speaking, two kinds of biological data: pathogenicity tests and bacteriophage susceptibility tests.

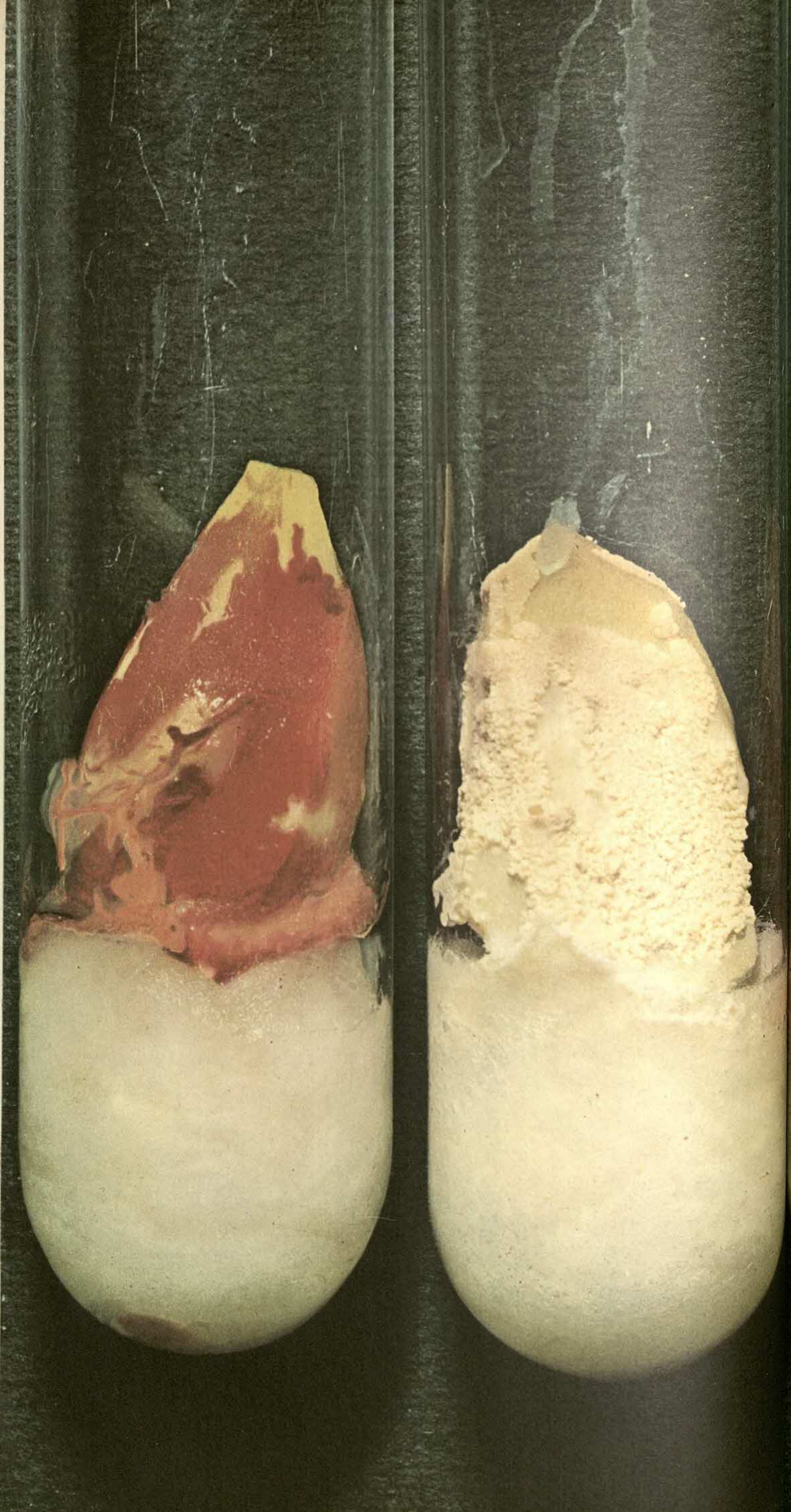
A germ which is thought to belong to a pathogenic bacterial species will be expected



Individual bacteria of the same species may show totally different forms. The picture shows the general appearance of a culture of the cholera vibrio, remarkable because of the simultaneous presence of spiral forms (below), bacillus forms (above), and in the centre of the field, spherical forms. (Istituto Superiore di Sanità, Rome).



The isolation and culture of bacteria require the use of different types of culture medium, according to the needs of the different species. Various incubation processes are used in order to obtain the characteristic colonies of chromogenic bacteria shown here. The medium is potato-glycerol agar. The cultures were grown for twelve days at 25°C. Above, from left to right: (1) an unidentified red bacillus isolated from the eggs of ants; (2) Bacillus aurantiacus tingitanus *(Remlinger and Bailly, 1935); (3)* Bacillus chlororaphis *(Guignard and Sauvageau, 1894). Opposite, from left to right: (1) an unidentified red bacillus isolated from the eggs of ants; (2)* Mycobacterium paratuberculosis, *a cream-coloured strain; (3)* Mycobacterium paratuberculosis, *a vermilion-coloured strain; (4)* Pseudomonas aeruginosa var. pyocyanigenes; *(5)* Serratia marcescens *(laboratory strain Ra.) (Photos Lod. From the laboratory of J. G. Marchal).*



to give rise in a receptive animal to distinctive morbid symptoms. Pathological material which contains pneumococci (for example, sputum), or the virulent cultures, when inoculated by subcutaneous injection into a mouse or rabbit, rapidly provokes a fatal septicaemia. The guinea-pig is very sensitive to anthrax infection; it dies two days after the inoculation with the germ, with manifestation of distinctive symptoms. Guinea-pigs and rats die within a few days when they have been experimentally infected with *Pasteurella pestosa*, and so on.

The bacteriophages[10] are viruses capable of destroying certain bacterial cells; possessing a specificity of action, they can be used for identification purposes. One cannot, however, say that a specific bacteriophage is able to act upon the whole of a bacterial species: some germs are sensitive and others are resistant. Within a species it is possible to distinguish different bacterial types precisely in relation to their sensitivity or resistance to different bacteriophages.

The example of meningococcus

The problem of bacterial identification can present itself under various aspects. One may wish to know whether a particular strain belongs to a particular species, or to identify an unknown strain, or to isolate and identify all the bacterial species present in any given material; but for each objective the same data are used. Familiarity with the key of classification to which one refers accelerates the work of identification.

Let us take, as an example, the bacteriological examination of pharyngeal mucus from a patient in whom presence of meningococci is suspected. It is known that this germ is very sensitive to desiccation, also that it does not grow on ordinary culture media or at room temperature, and that it is a strict aerobe.

With a piece of cotton wool some pharyngeal mucus is removed and immediately inoculated, using a standardized technique, on to some plates of serum agar or of blood agar warmed beforehand to 37°C. These plates are placed in an oven at 37°C; and after twenty-four hours colonies develop. Meningococci on such media give rise to isolated, circular, grey, transparent colonies about 2 mm in diameter. Thus all the colonies which show a different pigmentation and aspect can be discarded as a result of this test. If, however, one is afraid of omitting from the examination a colony of meningococci differing in appearance from the normal (and this may occur), the oxydase proof can be used.[11] For this, a solution of tetramethylparaphenylendiamine is poured on to the plate and then swiftly removed. The meningococci colonies and those of other bacterial species belonging to the same genus, *Neisseria*, are coloured a purplish red. A small fragment of each colony suspected, or which has given a positive response to the oxydase test, is put on a slide, stained according to the Gram method, and then examined under the microscope. All colonies composed of Gram-positive elements are eliminated, the meningococcus being Gram-negative. Next, all the colonies made up of Gram-negative micrococci (diplococci) are picked from the isolation plates and transplanted on to fresh media. After twenty-four hours' incubation at 37°C it is possible by further tests to distinguish among the colonies constituted by micrococci (diplococci), non-pigmented, strictly aerobic, nutritionally exacting, and Gram-negative, those which are formed of meningococci and those which are made up of related but different species. For this, two criteria are used: the ability to ferment sugar, and the serological reaction.

The meningococcus can ferment glucose and maltose, with the production of acid, but not gas, whereas the otherwise similar gonococcus can ferment only glucose. Other *Neisseriae* that resemble meningococci but are non-pathogenic, either do not ferment these two sugars or ferment them at the same time as the others.

Finally, serological tests are made: only the meningococcus reacts (by agglutination) to the action of anti-meningococcus serum. By additional testing against a range of sera, one can identify not only the species of meningococcus (*Neisseria meningitidis*), but also the type within the species.

(3) Strict aerobes *are organisms which can live only in the presence of oxygen;* microaerophils *are those which can live only under a reduced tension of oxygen;* strict anaerobes *are organisms that cannot live in the presence of oxygen.*

(4) Indicators: *substances which change colour according to the degree of acidity or alkalinity of the medium in which they are incorporated.*

(5) Amino-acids: *organic acids in which one or several atoms of hydrogen have been replaced by amino groups* ($-NH_2$).

(6) pH: *a negative decimal logarithm of the hydrogen ion concentration in a solution; pH 7 indicates neutrality. The lower values down to zero indicate increasing acidity, and values from 7 to 14 indicate increasing alkalinity.*

(7) Antigen: *a foreign substance which, when introduced into an organism by a route other than the alimentary canal leads to the formation of a particular substance called an antibody. This antibody reacts in a specific manner with the antigen that has induced its formation and with that only.*

(8) Immune-serum: *serum containing antibodies; for example, anti-typhoid serum contains the antibodies directed against the antigens of the typhoid bacilli.*

(9) Agglutination: *the joining together of bacterial cells in granules visible to the naked eye, due to the action upon them of the specific antibodies.*

(10) *See the chapter 'The bacteriophages' by P. Nicolle.*

(11) Oxydases: *enzymes catalyzing the oxidative processes.*

The culture of bacteria

Jean-G. MARCHAL

The maintenance of laboratory bacteria can be routine work. Many different culture media can be used, but when a particular aim is given to an investigation, certain media become preferable to others. Several examples are here described before we come to the study, properly speaking, of growth factors. Growth factors are well-defined substances, the absence of which prevents the microbes from reproducing. A large number of bacterial growth factors have been identified with animal vitamins, illustrating the unity which can be recognized in the general constitution of all living beings.

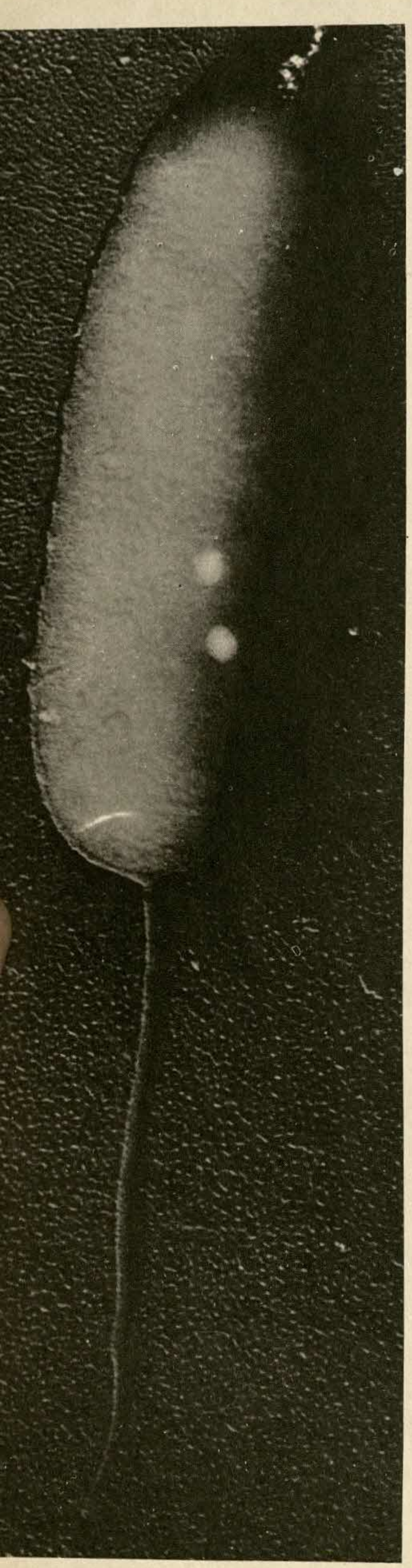
The modern bacteriologist who wants to study a bacterium always puts into practice the principles established by Pasteur; namely, he first isolates it from the complex medium in which it grows and then transfers it to a medium favourable to its development. The preparation of such nutritive substrates and their sterilization and correct preservation must be a constant preoccupation.

The preparation of culture media implies on the part of the bacteriologist a wide knowledge of the needs of different species and the means of satisfying them. In general, saprophytic organisms (non-pathogenic germs) that are able to live at the expense of inert matter of mineral or organic origin, have simpler nutritional requirements than parasitic organisms (pathogenic germs). The culture medium used daily in laboratories can be divided into empirical and synthetic types.

Empirical media

Empirical media, first used in Pasteur's time, are still very valuable for the culture, isolation and maintenance of pathogenic bacteria. They contain a nutritive base (usually meat extract) which provides substances such as nitrogen and carbon, proteins, amino-acids and mineral salts. Additive substances include peptone, sodium chloride (for the correct osmotic pressure), and phosphate salts which play the role of buffers,[1] that is to say, stabilizers of the pH of the medium.

(1) Buffers *are mixtures which strongly oppose any change in pH, whether by the addition of an acid or of an alkali. Most biological media possess a considerable buffering capacity. This contributes to the maintenance of a constant pH for the organisms.*



Agrobacterium tumefaciens *provokes cancers of very different appearance on plants into which it is inoculated. (see pp. 104–5). (Mag. × 50,000. Document I.N.R.A.).*

The empirical media are prepared most often from animal tissues, of which the important elements are rendered soluble by aqueous extraction or after chemical or enzymatic hydrolysis,[2] which liberates a large number of substances favourable to microbial growth.

This category of media includes the following types:

1. The commonly used meat broth or media enriched with albuminous liquids, bile, growth factors[3] (vitamins, globulin extracts, etc).
2. Selective media, more particularly adapted to the isolation and culture of certain forms.
3. Enrichment media—for encouraging predominance of a particular species existing in a mixture of organisms.
4. Differential media or media for identification—designed to study biochemical properties such as the production of indol, hydrogen sulphide, glucidolytic action, etc.

Simple empirical media in common use

The culture of bacteria was first made in liquid media and the most useful amongst these has been and still is an aqueous extract of meat, to which is added the product of the digestion of proteinaceous substances, namely peptone,[4] and which has been brought to pH of about 7 (see note 5). The substance thus constituted is usually called culture broth.

After its inoculation with a small number of bacteria, and having been left to stand for some time at a suitable temperature, growth is often very abundant, but it is not always entirely satisfactory. If several species are present in the bacterial specimen, a mixed culture generally results, or one species will very rapidly become predominant and cause the disappearance of others which may have been associated with it and which one wishes to isolate. It thus becomes difficult to study a species in pure culture.

To overcome the disadvantage of a broth medium, Mme Hesse, assistant of Robert Koch, suggested the addition to the liquid medium of an inert substance such as agar,[6] which causes it to go solid. By heating a broth with 2-3 per cent of agar, a jelly is formed which most conveniently remains firm at temperatures below 45°C, but which melts when the temperature is raised to boiling point.

To use this nutritive agar, the most usual method is to pour 15-20 cc of the medium, after liquefying it, into some flat glass containers (Pétri dishes). It is then allowed to solidify by cooling, and upon the surface of the gel is placed a small quantity of the specimen which one wishes to examine bacteriologically. This is spread out on the surface of the agar. (It is also possible to use the Koch method of isolation.)[7] After leaving for 24-48 hours in an oven regulated to a suitable temperature (37° for pathogenic bacteria, 25-30°C for saprophytes), colonies appear. These are visible to to the naked eye and contain millions of organisms.[8] These colonies are occasionally very characteristic and may allow a specialist immediately to distinguish species or even strains within a species.

Winogradsky, when studying certain bacteria in the soil, replaced the agar by silica gel in which he incorporated different nutritive substances, thus effecting biological conditions more favourable for the organisms found in nature.

Often gelatin[9] is substituted for the agar, to form a solid nutritive surface. It is incorporated in the medium under the same conditions as the agar and is very useful for revealing and isolating certain bacteria which produce proteolytic enzymes.[10]

Selective media

The purpose of these media is to allow an 'absolute dominance' and a much more rapid growth of the pathogenic bacteria which one is seeking to isolate within a mixture.

For example, lactose agar is very favourable to the growth of bacteria, such as the salmonella and shigella responsible for intestinal infections. In these media, antiseptics like bile salts are introduced (salmonella-shigella medium) and these allow the growth of the two species, whilst hindering the others. If, instead, a colouring matter, called brilliant green, and sodium tetrathionate are added (Kristensen's medium) the growth of salmonella is favoured and that of shigella suppressed.

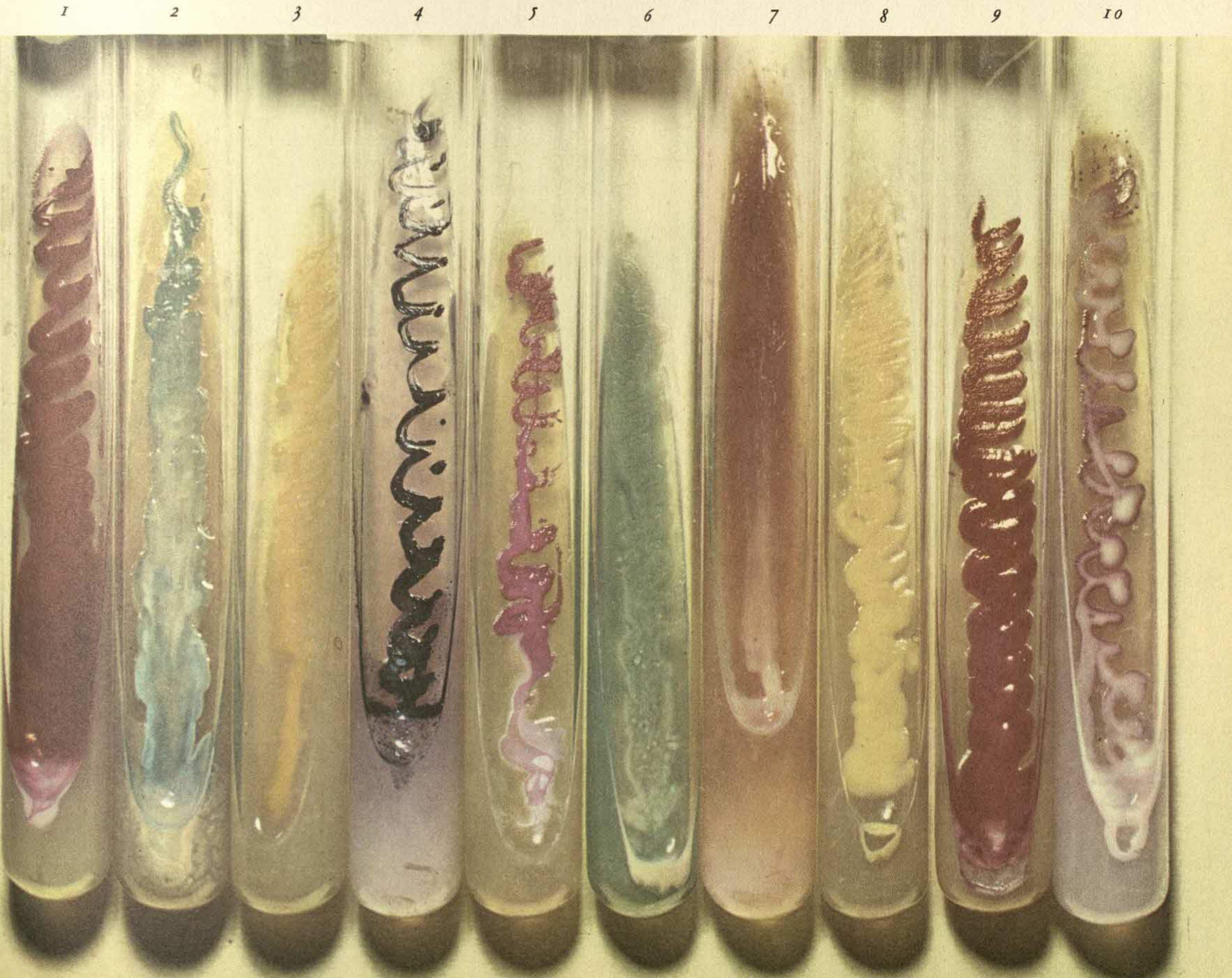
Enrichment media

These media allow an easier diagnosis of infections where germs are found in minute quantity in a pathological product. The medium of Müller-Kauffmann with a base of bile and of sodium tetrathionate, and that of

Culture of chromogenic germs

The identification of bacteria is greatly helped by growing them on culture media, the constant task of the bacteriologist. The bacteria illustrated here were obtained after six days of culture at 25°C on peptone agar with an admixture of glycerine potato broth. (Photo Lod. J. G. Marchal Laboratory).

1. Serratia marcescens *(Tobie strain)*
2. Bacillus Le Monnieri *(Lasseur, 1913)*
3. Bacillus aurantiacus tingitanus *(Remlinger and Bailly, 1935)*
4. Chromobacterium violaceum *(Schröter, 1872)*
5. Rhodococcus rhodocrous *(Zopf, 1891)*
6. Pseudomonas aeruginosa *(Schröter, 1872)*
7. Pseudomonas aeruginosa *(Erythrogenic variety)*
8. Staphylococcus aureus *(Rosenbach, 1884)*
9. Serratia marcescens *(Strain Ra of the laboratory)*
10. Serratia marcescens *(Strain 125 American)*



Leifson containing sodium selenite, make possible the preponderance of salmonella when they occur in the specimen in very small numbers, mixed with very large numbers of saprophytes.

Differential media

These are more particularly reserved for revealing properties useful in the identification of species. Certain among them even allow several characters to be observed at one and the same time. To facilitate the identification of enterobacteria, the medium of Hajna, as modified by Kligler, is used; in this medium the fermentation of lactose and of glucose and the production of hydrogen sulphide can be observed simultaneously.

For a long time bacteriologists were content to isolate and cultivate bacteria of medical interest on these empirical media, which came to be added to natural liquids such as milk or even urine, or else pieces of potato, or vegetable matter, with generally adequate results. However, certain bacteria do not develop in these conditions: (*a*) the gonococci, which require the addition to the broth of specific proteins found in the blood serum or the ascitic fluid of human beings; (*b*) the haemophils, which, as their name indicates, require haemoglobin.

To these requirements it may be necessary to add others related to the temperature of the development, the water content of the medium and the humidity of the surrounding air. Certain species require an atmosphere rich in carbon dioxide, others can live only in the complete absence of oxygen. This latter condition necessitates development in an empty container or in a medium containing reducing substances.

Synthetic media

It is by the use of chemically defined media, that is to say, media of which all the elements contributing to their composition are known, that the culture of bacteria has made the greatest progress. In 1870, Raulin, in memorable researches, showed how complex is the determinism of the development of a micro-organism in a synthetic medium. The medium which he invented is a model of its kind, and the method of application, at once synthetic and analytical, which he devised for its application, showed itself among the most profitable for microbiology and for the physiology of higher plants.

Lasseur, in 1911, advocated the use of synthetic media in the study of chromogenic bacteria, thus extending the work of Raulin. All research carried out since then has shown that the requirements in inorganic salts are very similar for all bacteria, which do not seem to have needs different from those of higher organisms.

The essential mineral needs of many micro-organisms have been determined by the chemical analysis of their ash. It has been found that an aqueous solution containing phosphates of sodium and potassium, magnesium sulphate and calcium chloride, at concentration from 0·05 M to 0·0001 M, is necessary. Other mineral elements at the trace level are equally indispensable. Molybdenum, for example, intervenes in the process of fixation of gaseous nitrogen by *Azotobacter*, at the concentration of 10^{-9} M. Iron at an optimal dose of 0·1 mg per litre superimposes the multiplication curves of the germ and the production of toxin by the diphtheria bacillus. Above this concentration, if the first curve continues to grow, the second diminishes.[11]

The metallic elements required at the trace level enter into the constitution of enzymes, which function in organisms as specific catalysts, or in the building up of metabolic pigments, such as the cytochromes normally produced by bacteria.

Knight considers that the mineral elements needed in culture media have three distinct functions: (*a*) to act as a buffer modifying the osmotic pressure of the solution or influencing the permeability of the cellular wall; (*b*) intervention in assimilation, as protoplasmic or cellular constituents; and (*c*) a catalytic action.

Synthetic media must also contain sources of carbon and nitrogen, as well as substances essential to metabolism called growth factors.

As far as carbon is concerned, the autotrophic bacteria can use substances such as carbon dioxide, whereas the heterotrophs require organic substances. Those which can be utilized are very numerous and differ very widely according to the microbial species envisaged.

Nitrogen metabolism varies from one bacterium to another. Some species find the nitrogen needed for their development in mineral nitrogen compounds or even in nitrogen gas; other species require amino acids or even proteins which they decompose by the action of proteolytic enzymes.

Tryptophane, which is one of the most important amino acids in protoplasm, can be synthesized by certain bacteria which therefore can grow on a medium which does not

(2) Chemical hydrolysis: *an operation which consists of heating a substance to a high temperature after the addition of an acid or an alkali.* Enzymatic hydrolysis *is frequently used in biological studies in the place of chemical hydrolysis. It is brought about by adding enzymes (such as pepsin, trypsin, etc) to the substance to be broken down.*

(3) *A* growth factor *is a substance which an organism cannot itself synthesize, but which it needs for its own multiplication.*

(4) Peptone: *produced by the digestion of proteinaceous substances of vegetable or animal origin.*

(5) *In order to simplify the idea of acidity, Sorensen proposed expressing it not by the absolute concentration of hydrogen ions but by the number of zeros to the right of the digit 1, which one finds in the denominator of the decimal fraction representing this concentration. The pH symbol is, in short, merely the logarithm to base 10 of the reciprocal of the concentration of hydrogen ions. For a neutral reaction the pH is 7, and it is below this value for acidic reactions and above it for alkaline ones.*

(6) Agar: *a complex carbohydrate extracted from marine algae and often used for solidifying bacteriological culture media.*

(7) *In the* Koch method *the pathological specimen is treated to successive dilutions in liquid media; drops from the dilutions are then placed in tubes containing previously melted peptone agar, maintained at 50°C and then run out into Pétri dishes.*

(8) *For undertaking accurate work on cultures which must have a single bacterium for origin, Fonbrune's apparatus for the isolation of units is used.*

contain it; whereas others, such as certain strains of typhoid bacilli, can be made to derive it from ammonia, which constitutes the only nitrogenous element of a synthetic medium.

For growth factors, there are degrees of need. Thus Lwoff distinguished: (*a*) growth factors that are essential for cell multiplication; (*b*) those acting as stimulants, accelerating or ameliorating growth but not indispensable to it; (*c*) those concerned in adaptation to new conditions of life imposed on the bacteria; (*d*) those necessary in certain culture media; and (*e*) conditional stimulants which increase the development of the germ.

With all these requirements in mind, it is of interest to inquire how the bacteriologist proceeds to make a synthetic medium adapted to the development of a particular organism.

To reach this goal, two methods are employed: analysis and synthesis. In the first, he endeavours to characterize the different substances which appear in a medium of determined constitution in which the species under study will be developed in well-established conditions of temperature, aeration and time of culture. The identification of these substances in cultures of different ages allows him to reconstruct and link up the stages in the metabolism of a bacterium.

In the synthetic method, the bacteria are cultivated on a medium of known composition, which is progressively diminished until each of the medium's constituents is suppressed in succession. In this way the effect is determined of the presence or absence of a trace element or growth factor on the weight yield of microbial bodies or on certain metabolic functions.

(9) Gelatin: *a substance having the appearance of a jelly and obtained from the fibrous tissues of animals.*

(10) Enzymes: *substances with catalytic action allowing the development of biochemical reactions in the conditions peculiar to the cell and to the environment in which it lives.*

(11) *1/100,000 of iron added to Sauton's medium triples the growth of Koch's bacillus. Salts of manganese are necessary for the optimal growth of tetanus bacilli.*

Bacteria in the service of biology

The observational science of microbiology first came to be linked with the biological disciplines when Pasteur demonstrated the role of microbes in fermentation and putrefaction, which up to then had been considered mysteries.

Even at the beginning of the twentieth century, research workers were still preoccupied with the descriptive study of pathogens and agents of fermentation, although they had, of necessity, to define some properties which seemed to them useful for the identification of species. Then the study of bacteria[1] was very rapidly revealed to have a didactic value and a considerable biological significance. Special techniques, continually being perfected now, make it possible to verify hypotheses and provide solutions of biological problems that cannot be tackled in other ways.

Problems of structure and physiology

The most recent techniques in physics (phase contrast and electron microscopes) have confirmed the existence in bacteria of constitutive elements in regard to every cell, and have brought more precise knowledge about their structure.

The cell is bounded by the cell wall, a thin enveloping structure (thickness 10-20 mμ) that maintains the specific form and maintains its internal tension and selective permeability. This membrane varies in composition between species; it is the site of the Gram-staining reactions[2] and allows the distinction of Gram-positive bacteria from Gram-negative bacteria. It is also the site of acid-alcohol-resistance observed in the mycobacteria. The biochemical explanation of these staining properties is as follows.

The Gram-positive reaction is due to the existence in the cell wall of a complex of magnesium ribonucleate, ribonucleic acid and a polysaccharide. These can be extracted from the bacteria by the action upon them of a solution of sodium cholate at 60°C, and replated afterwards, in certain conditions, as demonstrated by Stacey (1950) on cells reduced to the state of cyto-fragments which regain their Gram-positivity[3] in this way. The factor for the acid-alcohol-resistance[4] is lodged in the waxy coating of the mycobacteria.

Certain species are characterized by a

(1) *Far from being distinct and separate from the animal and plant worlds,* bacteria *have come to be integrated in the world of all living beings. Relatively simple in structure, they have helped to elucidate problems of great complexity.*

(2) *A bacterium is called Gram-positive when, treated successively with gentian violet and Lugol's iodine (iodine in potassium iodide), it retains a violet-black colour after being washed in 95° alcohol. Treated under the same conditions, a Gram-negative bacterium is decolorized.*

(3) *Salton (1953) showed that lipids are more abundant in Gram-negative bacteria, which also contain certain amino acids and a greater quantity of magnesium.*

(4) *The mycobacteria, treated with fuchsin (Ziehl) when heated, are coloured an intense red. The red-coloured complex is insoluble in alcohol and diluted nitric acid. These bacteria are therefore referred to as being* acid-alcohol-resistant.

sticky capsule which makes up the external layer of the wall. This is seen, for example, in type III *Diplococcus pneumoniae* and is of great interest. Composed of a polysaccharide of high molecular weight and of a small quantity of polypeptide, it renders this pneumococcus more resistant to phagocytosis. Heidelberger (1947) has isolated in a pure state the polysaccharide constituent of this capsule, and he has shown that it is strongly antigenic[5] for man.

Within the cell wall the cytoplasm is enclosed by a thin membrane (up to 40 Å wide in the coli bacilli). Certain authors consider this cytoplasmic membrane as constituted essentially of mitochondria and of cytochromes, the former useful for nutrition and the latter for respiration. Besides these enzymatic functions, the cytoplasmic membrane is active in selective cellular permeability, as has been demonstrated by the study of bacterial protoplasts. The cytoplasm plays a primordial role in the synthesis of the proteins of bacteria. The physiological role of its inclusions is still the subject of discussion.

The cytoplasm in certain species contains pigments that may be supported by structures comparable to the chloroplasts of plants; these are useful for respiration. Others have an imprecise significance, for the bacteria which have lost the capacity to produce them develop as well as those which still possess them.

The reserve substances observed in the cytoplasm are numerous: glycogen and fats, enclosed in vacuoles of various shape, and in certain species, sulphur in a colloidal state, iron in the form of inclusions or impregnations, etc.

Without knowing exactly what is the site of their formation, it can be said that bacterial toxins are derived physiologically from enzymes and from cytochromes.

Metabolic problems

Generally speaking, metabolism can be defined as the group of chemical changes taking place within living beings and which are responsible for their growth. Some of these changes are catabolic and consist of the wear of carbon chains with the liberation of chemical energy; others are synthetic or anabolic, and consist of the building up of complexes from the simplest elements. Work on bacterial metabolism has shown that respiration is a process of dehydrogenation by which a series of conveyors of hydrogen (or of electrons) come into play, in succession.

During the period between 1920 and 1940 physiologists and biochemists discovered the existence of chemical compounds and metabolic reactions in micro-organisms that were strictly analogous to those found in the cells of higher organisms. They thus demonstrated a common pattern of cellular nutrition and began to see the possibility of cultivating and continuously maintaining strains of animal cells in semi-synthetic media. Already there have been some spectacular results in the study of viruses. The time is perhaps not far off when, by means of our knowledge of microbes, we shall be able to make out the 'biological specification' of each type of cell, human or animal, and it will be possible either to preserve in descendants the original characteristics or to modify these by producing deficiencies in a given metabolite.

The study of metabolism in terms of the power of synthesis allows the following kinds of bacteria to be distinguished:

1. *Autotrophs:*[6] these bacteria need only inorganic substances, from which they are capable of synthesizing all their essential metabolites.

2. *Heterotrophs:* these bacteria, on the contrary, obtain from the external environment certain essential organic substances, for they have lost, in varying degree, the capacity of making them.

3. *Prototrophs:* these organisms are heterotrophic in respect of organic carbon chains and cover their nitrogen needs by fixing nitrogen from the air.

Among the latter are the rhizobiums found in the root nodules of leguminous plants. They can fix and thereby make use of atmospheric nitrogen. Their study has led to a more precise understanding of the curious mechanism of symbiosis and they are of great interest agriculturally.

The cycles of biogenetic elements such as those of carbon, nitrogen, sulphur and phosphorus, are now well established. The use, in the laboratory, of marker elements and of modern methods of research, allows one to follow much more easily the syntheses carried out by the bacterial cell.

Problems of energy

All bacteria need a source of energy for the building up of their structures. Autotrophs use light or chemical energy. One group (Chlorobium) possesses a pigment (bacteriochlorophyll) that plays a role analogous to that of chlorophyll in plants. Others, such as *Thiobacillus* and *Nitrobacter*, obtain the energy

(5) Antigenic *ability is the power all 'foreign' protein possesses of giving rise to antibodies in the animal or man into which it is injected.*

(6) Autotrophic *bacteria, like green plants, receive the carbon and nitrogen they need in the form of simple inorganic substances like* CO_2*, ammonia, nitrates or nitrites. The heterotrophs cannot synthesize certain molecules, having progressively lost their autotrophic capacity. They must obtain their carbon and nitrogen from foods made by other beings and on which they are entirely dependent. An example is the pathogenic bacteria difficult to culture outside the organism.*

(7) *All synthesis requires energy for its realization. The living being must have at its disposal an external energy* (exergonic reactions) *in addition to its internal energy* (endergonic reactions).

(8) *Gale (1956) showed that staphylococci disintegrated by ultra-sonic vibrations are incapable of growth and respiration, but can still assimilate amino acids if care is taken to add to the culture medium some energy-giving substance such as adenosine-triphosphate (ATP).*

(9) *If the S forms of these organisms are considered more pathogenic than the R forms, it does not mean that they are more active biochemically but only that they are more specialized.*

(10) *'Bios', a growth factor contained in yeast extract and discovered by Wildiers in 1901, was found by Eascott (1938) and Posternak (1942) to be a mixture of meso-inositol and biotin (Kögl, 1936). At present it seems probable that bios provides an explanation for the dispute between Pasteur and Liebig. Both attempted to grow yeast in a medium containing only mineral matter and sucrose.*

Continued on page 66

they need in the oxidizing reactions of mineral compounds, in the transformation of sulphides into sulphur and sulphates, of nitrites into nitrates. Heterotrophic bacteria use the breakdown of organic substances by a curious mechanism of coupling endergonic and exergonic reactions.[7]

The energy is not, however, freed rapidly, for too large a contribution would be useless, and the return which would result from it would be too weak. The breakdown of glucose, for example, passes through well-known intermediate stages before reaching carbon dioxide and water. It is now known that, through the intermediary of hexose phosphates, glycogen is converted to pyruvic acid, a centrally important compound in metabolism, the breakdown of which is carried out in the citric acid cycle.

In breaking down the molecule of glucose, the bacteria find step by step the energy they need and store it in the form of compounds which can be thought of as energy reservoirs. In this storage, which varies according to the species, phosphorus occurs in compounds[8] possessing high energy bonds, that is to say, compounds that by hydrolysis can release this energy whenever the cell needs it.

Enzymological problems

If by ingenuity we have succeeded in reproducing certain metabolic processes, using bacterial, plant or animal extracts, the cell jealously keeps to itself the fine details of the natural mechanisms it uses to attain its ends. However, the biochemists have succeeded in isolating in a fairly pure state enzymes which direct cellular metabolism.

Bacterial enzymology is one of the most explored fields in general enzymology because of the simplicity of the techniques that can be used; it is only necessary to put a bacterium into a suitable substrate to witness the appearance of the enzyme which will allow its transformation.

It is through the intervention of a succession of specific enzymes, which guide and launch cellular activities, that cellular metabolism proceeds, one of these enzymes preparing the ground from which the next will arise. Karström has distinguished constitutive and adaptive enzymes. The former are those that the bacterium has independently of the environment in which it finds itself; the latter are produced on demand when the culture medium contains particular substances. Evidently there must exist in the bacterial genotype rudiments or a tendency towards the formation of such adaptive enzymes.

The bacterial cell has great adaptability, but naturally there are limits for each species, limits fixed round a constructive plan that is genetically stable. In support of this idea, Deotto has shown that in certain coli bacteria there are smooth (S) and rough (R)[9] colonies which differ in their utilization of certain substances. The smooth form is always biochemically more active than the rough form but, besides the S and R variants, there are numerous sub-variants, from the point of view of metabolism, which argue in favour of fluctuation around a fundamental type, of selection and fixation of certain among them under the influence of environmental conditions.

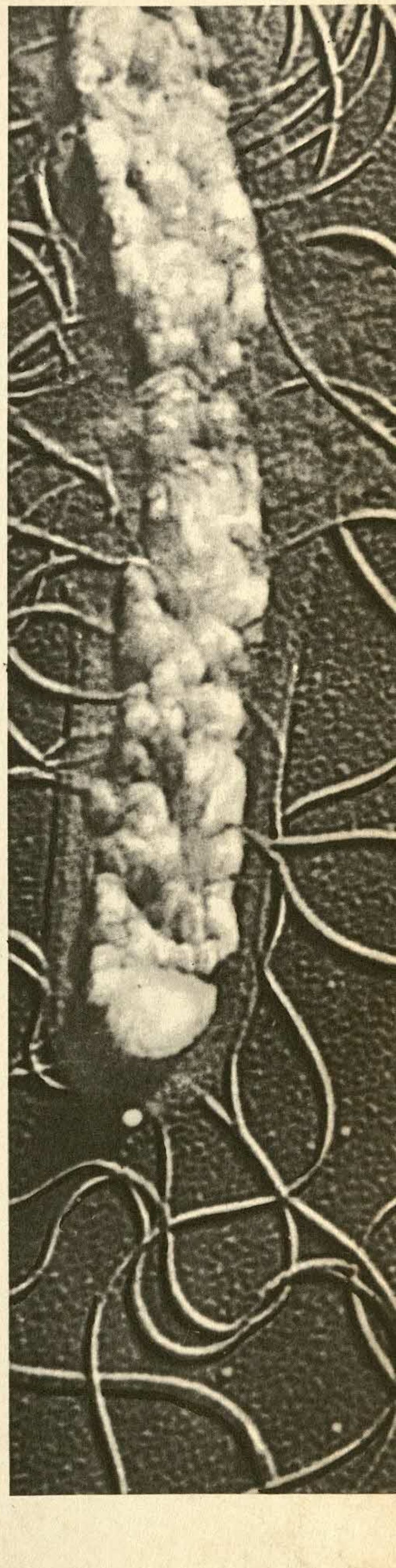
Problems of growth factors

The culture of bacteria in defined chemical media has led to a much clearer understanding of the nature of growth factors. These are substances considered as essential metabolites that cannot be synthesized by some micro-organisms. Growth factors for microbes are the equivalent of vitamins for animals; though certain of them are unknown outside bacteria, they nevertheless play the role of vitamins in animals.

A growth factor is defined for a specific organism when in the absence of it all multiplication is impossible; it may be necessary only in certain conditions of culture[10] and is therefore different from a stimulant or a factor of adaptation.

Pfeiffer (1893) first showed the existence of these substances. He established that *Haemophilus influenzae* can develop aerobically only in the presence of blood. Davis, in 1917, demonstrated that the blood contained two factors, called the X and V factors by Thjotta and Avery (1921). The work of A. and M. Lwoff (1936) showed in detail their chemical nature and role.

The discovery of the requirements of haemophilics for factors X and V has thrown interesting light on the mechanism of satellitism and symbiosis.[11] Grassberger (1897)



Examination under the electron microscope allows the analysis of structures formerly invisible and is helpful in the classification of species. This bacillary form of Clostridium tetani *reveals perfectly the cytoplasmic insertion of the cilia. (Mag.× 40,000. Istituto Superiore di Sanità, Rome.)*

observed that the seeding on blood agar of a mixture of *Haemophilus influenzae* and *Staphylococcus aureus* allowed beautiful colonies of the former to be seen in the neighbourhood of the colonies of the latter, whereas at a distance they were scarcely visible to the naked eye. The explanation of this phenomenon is simple. The *Staphylococcus*, unlike *H. influenzae*, makes co-dehydrase in large quantity. This factor easily diffuses in the medium, and is carried to the *Haemophilus*, which finds in its neighbourhood a very large quantity of an indispensable metabolite and is able to grow rapidly.

Some kinds of symbiosis can equally well be considered as an 'ecological sequence of the necessity of growth factors' (Lwoff 1943). Thus *H. canis* needs the factor X, and *A. parainfluenzae* needs the factor V. If these two organisms are seeded separately on peptone water, they do not develop, but together they both produce abundant cultures, each giving to the other the indispensable metabolite.

The mechanism of action of growth factors is extremely varied. Vitamin B_2 plays the role of hydrogen carrier; the phosphoric ester of pyridoxine is the co-enzyme of certain amino acid decarboxylases and transaminases; and vitamin B_{12} has considerable importance in the synthesis of deoxyribonucleic acid.

Our knowledge of the chemical functions exerted by these different substances has led to the discovery of numerous products with chemotherapeutic activity whose role is now better understood.

Substances which destroy bacteria by inhibiting certain of the metabolic processes have been synthesized. The anti-metabolite idea became important when Woods (1940) demonstrated that the anti-sulphonamide activity of yeast extracts was due to para-aminobenzoic acid. In comparing the structure of this substance with that of p-aminophenyl sulphonamide, Woods, in agreement with Fildes, put forward the hypothesis of a competition by analogy with spatial structure. At the level of enzyme systems, sulphonamide would be substituted for p-aminobenzoic acid (vitamin H), an essential metabolite for bacteria, and would thus act as an anti-metabolite.

The list of anti-metabolites is increased by the numerous antibiotics discovered almost every day and the concept of autogenism by structural analogy, born in bacteriology, has already been transferred to pharmacology, where Meunier (1942-3-7) and Mentzer (1950-4) have prepared anti-coagulant substances with a similar molecular structure to phtiocol, having properties like vitamin K. Folic acid[12] may also be mentioned in this connection. This substance is concerned in the synthesis of nucleic acids and has chemical affinities with para-aminobenzoic acid, which also makes it an anti-sulphonamide.

Because certain bacteria cannot be grown in the absence of a specific metabolite, the idea arose of using them for detecting and determining the quantity of B-group vitamins, which are for them factors of growth. Patient research has established the specific microbial types whose rate of growth is proportional to the quantity of growth factor added to the synthetic medium; Lwoff (1939) showed that the development of proteus was directly related to the quantity of nicotinic acid or amide present in the medium.[13]

Although it is only within the last twenty years that the microbiological quantity determination of vitamins has acquired all its importance, the microbiologist Raulin as early as 1870 used micro-organisms to indicate certain mineral or other deficiencies in soil.

The method has general application to other elements: molybdenum, essential for the development of those micro-organisms which fix nitrogen and are found in the nodules of leguminous plants; manganese and zinc, which form part of the composition of certain metallo-protein enzymes; magnesium, which intervenes in the synthesis of carboxylase; and iron, which is a constituent element in a range of functional substances found in bacteria and in other living beings (cytochrome, peroxydase, cytochromeoxydase, catalase).

In 1944, we ourselves made, from a colourless precursor of a pigment produced by a chromogenic bacterium in a synthetic medium, a biological indicator of great sensitivity for iron.

The study of soil bacteria has allowed us to extend our knowledge of the synthesis of many organic substances, and it has equally led to a better understanding of the changes that the microbe performs on animal or plant organic matter in the process of remineralization.[14] Controlled fermentations have led to the production of hydrocarbons such as methane (marsh gas) that can be used as a fuel. Petrol has already been synthesized in the laboratory, by using the enzymatic mechanisms of bacteria. These researches have only a curiosity value perhaps, but they are of great interest, for they show that some bacteria, placed in optimal conditions for particular activities, can in a few weeks produce materials which nature has taken centuries to prepare and store.

Continued from page 64

Pasteur was successful, possibly because he inoculated his media more abundantly than Liebig, and perhaps carried over into the medium sufficient bios arising from dead cells contained in the inoculum for normal growth to take place.

(11) *The thermostable factor X has been identified as haematin. A. and M. Lwoff proved that the irreplaceable metabolite was the porphyrin fraction of the haematin, to which the bacteria could join iron to produce the co-enzyme of a cytochrome oxydase. Factor V, less thermostable, has been identified by the same authors as co-dehydrase, a normal constituent of a cell, which also plays a part in hydrogen transport.*

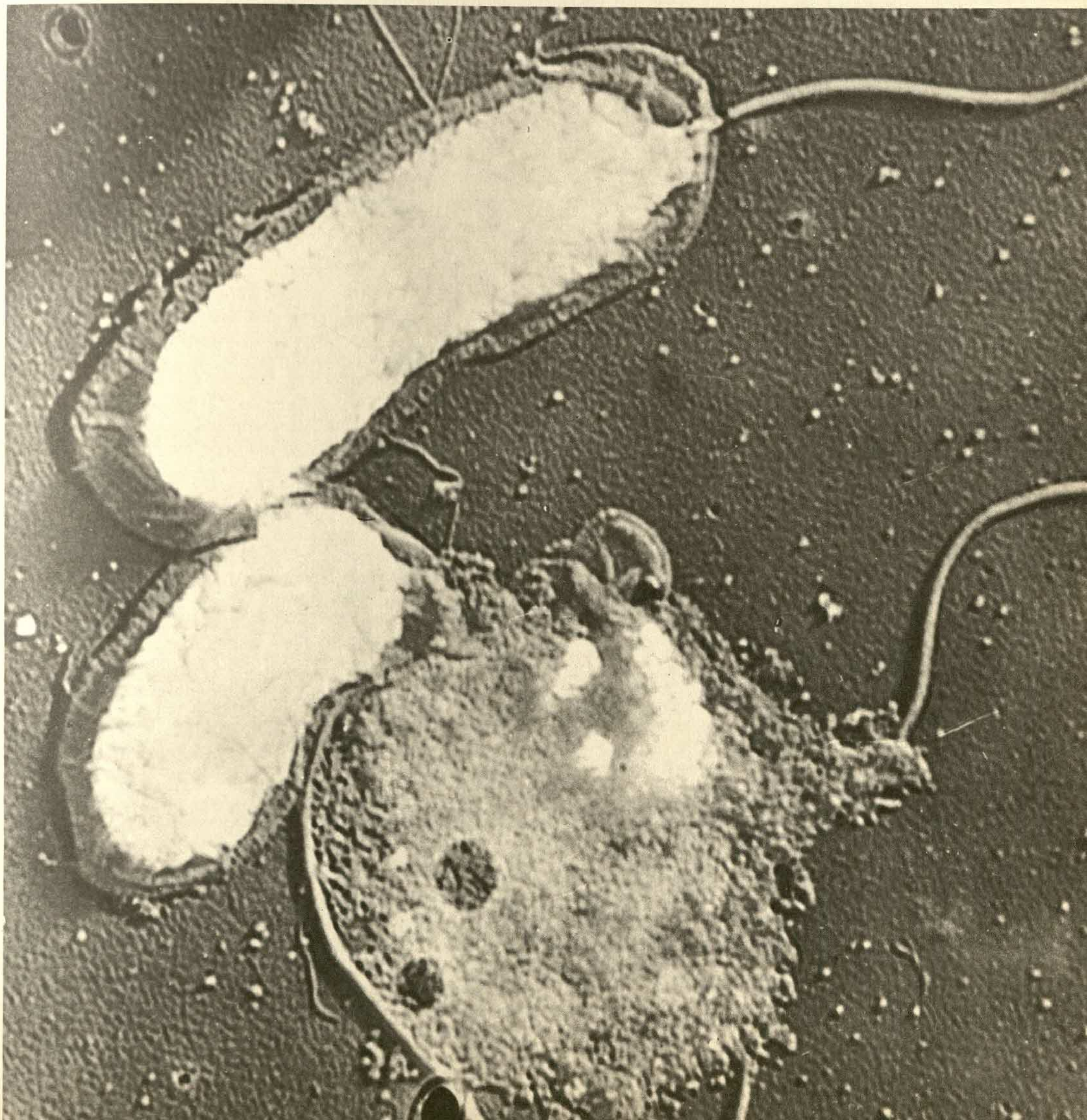
(12) *In the battle against cancer, researchers have tried to find structural analogues of folic acid to block the excessive synthesis of certain nucleoproteins. Among 13,000 synthetic compounds that have been examined, aminopterines, acting as anti-metabolites, have been the most active on mouse sarcoma.*

(13) *The rate of bacterial growth, which in a certain measure is proportional to the quantity of growth factor in the medium, can be ascertained by measuring either the cloudiness, or the amount of a chemical substance elaborated in the course of growth (lactic acid, for example, in the case of* Lactobacillus casei) *or the gas produced, or by weighing the mass of micro-organisms formed, as proposed by Schopfer, making* Phycomyces blakesleeanus *a test of the quantity determination of vitamin* B_1 *(1937–8).*

(14) Mineralization *consists of giving plants the indispensable chemical elements which they have drawn from the soil and the atmosphere. Without these micro-organisms animal and plant life would very rapidly become impossible.*

PART TWO

Activity of bacteria



Pathogenic bacteria: cholera vibrios (mag. ×31,500, Istituto Superiore di Sanità, Rome).

The role of saprophytic bacteria

Carlo ARNAUDI

The waste left by living creatures after assimilation of what is suitable for their nourishment, the remains of dead animals and plants on the ground or in water, are all promptly broken down by the action of microbes and reduced to their simplest constituent elements. It is true that among all these multiple interlocking chemical reactions there are many details which are still unknown to us, but some of the mechanisms are well understood, as is the nature of the micro-organisms which play the chief role in bringing them about.

Microbes form the keystone of the closed cycle which connects dead and living matter. The continuation of this cycle and the formation of new life depend on the breakdown of the corpses of animals and the remains of plants into simple inorganic materials, or at least into smaller structural units than the chemicals of which they are composed.

From a general point of view, all micro-organisms, whether pathogenic (that is, capable of causing disease) or not, exercise their activity in the direction of breakdown. The pathogenic ones are able to attack living tissues, whilst those which we call saprophytes attack only dead organic substance. Living tissues which are not capable of defending themselves successfully against the attack of parasitic bacteria quickly succumb and are then immediately attacked by the saprophytes.

Animals and plants obtain the chemical materials necessary for their development from their environment. Part of this material is passed on to other living organisms as food, part is set free in the course of exchange between the organism and the environment, but the most important part remains stabilized so as to make up the frame of plants and animals, even after their death. If the dead bodies of animals and the remains of plants were not broken down, if the mass of living organisms upon earth were appreciably constant, there would accumulate in the course of millenia so much organic material that life would become impossible. But, in fact, the remains of plants and animals are promptly broken down so as to be like the earth from which they obtained their food.

The action of micro-organisms

The importance of micro-organisms in decomposing complex organic substances and liberating elements can be seen from a consideration of the distribution of carbon dioxide. This gas is assimilated by plants in photosynthesis and built up into complex carbohydrates. It is estimated that the amount of carbon dioxide to be found free in the atmosphere at any one time is about four hundred million tons. That bound in the form of the organic materials of plants and animals, on the other hand, is about three thousand million tons, expressed in each case as simple carbonic acid. It can easily be seen that if the process of breakdown of organic materials which liberates free carbon dioxide were to be stopped for any length of time, the atmospheric reserve would soon be used up and photosynthesis would come to a halt. Happily for the continuation of life, most micro-organisms do not contain chlorophyll and must therefore obtain the energy which they require for their own needs by the breakdown into simpler compounds of pre-formed organic material.

The biochemical activity of micro-organisms is in general more potent than that of animals or plants. Thus, one gram of the *Micrococcus ureae*, containing about fifteen thousand million cells, is capable of decom-

HISTORICAL NOTES

1776
VOLTA *discovers marsh gas (methane), which is inflammable, in the reed-beds of Lake Maggiore.*

1838
BOUSSINGAULT *suspects that leguminous plants are capable of fixing atmospheric nitrogen.*

1860
PASTEUR *establishes that the decomposition of urea with the formation of ammonia is due to the action of micro-organisms.*

1864
These observations of PASTEUR *are confirmed by* VAN TIEGHEM.

Continued on page 72

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(1) Mummification: *the slow drying of tissues, not followed by putrefaction.*

(2) Pectins: *substances formed by the condensation of certain types of monosaccharides (sugars containing carbon, hydrogen and oxygen). They are widespread in the plant kingdom, forming the cement between plant cells.*

(3) Amino acids: *organic acids containing amino groups (—NH_2). Proteins are made up of amino acids.*

posing up to 1,200 grams of urea per hour. The extent of the activities of microbes might readily be deduced from the very large ratio of surface area to body weight which they present, since this, of course, means that there is a very extensive area of contact between them and the substance which they are attacking. Another fact which is not immediately obvious from a consideration of microbial phenomena, but which remains altogether significant, is the surprising speed at which motile bacteria are able to move. One of these may cover, on the average, twenty times its own length in a second, whereas a racehorse for example, manages to cover only eight times its own length in the same time.

Thus micro-organisms accomplish their work of decomposition mainly by attacking complex organic molecules and reducing them, through a series of intermediate stages, to a molecular structure still more simple, which reunites even the primitive gaseous forms, that is to say, hydrogen, oxygen, nitrogen, water vapour, hydrogen sulphide, carbon dioxide, ammonia, etc. The breaking-down includes complex organic molecules containing potassium, sodium, calcium, nickel, iron, zinc and copper, the metallic parts of which are converted into inorganic mineral salts.

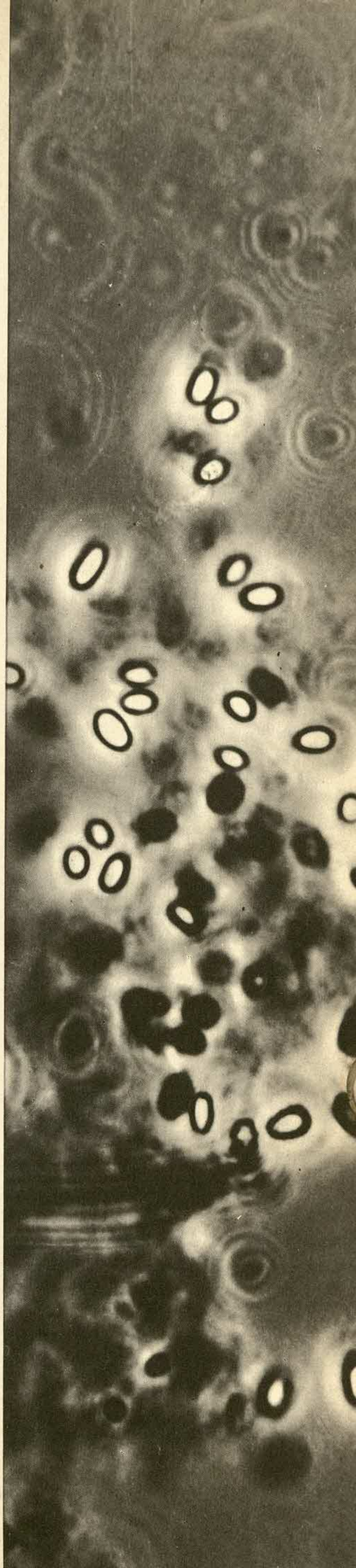
The most important element in the environment in which micro-organisms are to be found is provided by the particular organic substrate they decompose, but their activity is also influenced by such circumstances as the degree of humidity or temperature. It is equally the conditions such as those of intense cold or of high temperature, precisely because they inhibit all existence and development of microbial life, which determine the preservation of dead animals and plants throughout centuries. Frozen bodies of prehistoric animals are still found in a state of perfect preservation in the polar regions; and in desert areas under conditions of high temperature and low humidity, remains of animals or plants often undergo mummification[1] rather than decomposition. In both these cases the conditions are quite unsuitable for the growth of bacteria.

The role of micro-organisms in the maintenance of life on the surface of the globe, in other words the process of reduction of organic substances to inorganic ones, is nevertheless conditioned by factors of the environment which themselves regulate the distribution and reproduction of the micro-organisms. On the other hand, they show very different behaviour according to the surrounding physical factors. For example, in conditions of extreme cold we may find psychophilic (cold-loving) bacteria such as *Bacterium phosphorescens*, which is present in the Arctic ice, and, at the other extreme, there are organisms such as *Bacterium Ludvigii* for which the optimum conditions for development are temperatures of about 50°-60°C.

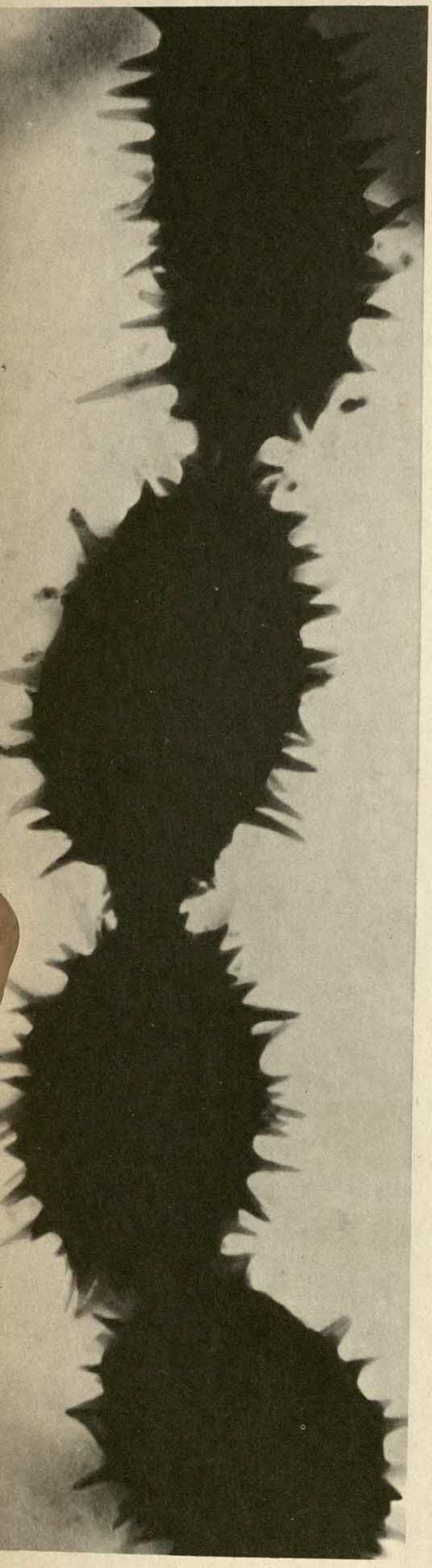
The breakdown of organic substances

The decomposition of the organic substances which make up the bodies of animals and plants is brought about by micro-organisms, some of which act in a very specific manner, while others have the power of attacking many different types of substances. The lower fungi and the moulds are in general endowed with a rich and varied enzymatic make-up which makes it possible for them to attack carbohydrates, even such complex ones as cellulose, as well as proteins and fats. For example, cellulose can be attacked by several species of fungi belonging, amongst others, to the genera *Aspergillus Penicillium*, *Fusarium*, *Cladosporium*, and *Trichoderma*, which act simultaneously on the pectins,[2] which are the cementing substances found binding together the fibro-vascular bundles of plants, as well as fats and proteins which are present in very small quantities in decomposing vegetable matter. Along with these organisms, and often intimately associated with them, we find on the other hand species of schizomycetae which have a pronounced specificity for cellulose; such micro-organisms can be cultured *in vitro* on pure cellulose and very quickly break it down. Those which act in this way on contact with air include the genera *Cytophaga*, *Cellvibrio* and *Cellfalcicula*, and in addition there are the clostridia *Bacillus methanigenes* and *Bacillus fossicularum*, which are found in marshes or in the depths of the soil and are capable of breaking up cellulose under anaerobic conditions, that is, in the entire absence of oxygen.

The simpler carbohydrates such as sugars and starches, and their derivatives, are attacked by many micro-organisms which ferment them and turn them into alcohols and organic acids such as lactic, oxalic, acetic, formic and butyric acid.



The collective species Bacillus subtilis, *of which the spores are shown here, includes some ninety sub-species which actively attack proteins and break them down. This activity is of considerable importance. (Mag.* × *2,000. Photo Arnaudi).*



The enzymes present in the spores of Streptomyces enable them to act upon carbohydrates as well as on the proteins and fats which are present in all vegetable matter. (Mag. × 50,000. Photo Baldacci, University of Milan).

Along with the decomposition of cellulose, that of the lignins, found in wood, is carried out exclusively by lower fungi, whilst the pectins are attacked more or less specifically by numerous schizomycetae.

The hydrolysis of proteins is carried out by innumerable bacterial species which are distinguished from one another above all by the degree of the breakdown they are able to bring about. In certain cases this breakdown goes as far as the liberation of ammonia and hydrogen sulphide, whereas in others it may stop at an intermediate stage, but conferring in each case, nevertheless, on the material attacked, macroscopic and organoleptic characteristics such as to define the group as a process of putrefaction. Sometimes, on the contrary, the breakdown is accomplished by certain species of bacteria which act simultaneously on the carbohydrates which may accompany the proteins. In such cases the increasing acidity of the surrounding medium, resulting from the fermentation of carbohydrates, confers upon the process those characteristics (for example, the absence of an unpleasant odour) which distinguish it from ordinary putrefaction. Thus we can distinguish between the simple proteolytic micro-organisms characteristic of putrefaction, such as *Clostridium lentoputrescens* and *Bacillus putridus*, and the mixed-type organisms which also attack sugars such as *Clostridium septicum*, *Clostridium chauvoei*, *Closteridium sporogenes*, etc. There are also many aerobic spore-bearing bacilli which attack proteins, but it suffices to mention the collective species *Bacillus subtilis* and *mesentericus*, comprising about ninety individual species, *Proteus vulgaris*, *Pseudomonas fluorescens*, and *Serratia marcescens*, better known as *Micrococcus prodigiosus*. The breakdown of fats is brought about by the action of many of the lower fungi, as well as by various species of schizomycetae. The breakdown due to lipolytic micro-organisms is brought about by the hydrolytic splitting of fat which yields glycerol and a mixture of fatty acids; the glycerol may be attacked in its turn by different micro-organisms of which *Bacterium glycerini* is one.

Animal and vegetable tissues contain other groups of substances like the steroids and glucosides which are also broken down, notably by the various lower fungi, in the surrounding environment.

Interaction of microbial species

Microbial species can in some cases develop concomitantly with others in varying degrees of interaction, which may go as far as symbiosis, each bringing advantages to the other; or they can show, on the other hand, phenomena of antagonism or reciprocal intolerance between two species. As an example, bacteria which decompose carbohydrates with the formation of acids and alcohols, even temporarily, can modify the environment so that it is unfavourable for the growth of those species which prefer alkaline conditions. Further, these same biochemical activities of different microbial species may give rise, even for the time being, to the appearance of organic substances possessing special physico-chemical properties such as a high surface tension, and these will of course be inhibitory to any species which is particularly sensitive to variations in surface tension.

Finally, species which give rise to antibacterial substances have the power of limiting or preventing the growth of other species which are susceptible to the action of these factors. There are many examples: we may mention the antagonism between *B. anthracis* and *B. mesentericus*, between *Pseudomonas aeruginosa* and *Staphylococcus aureus*, between the lactic-acid bacteria and *Clostridium putrefaciens*, between *Serratia marcescens* and various clostridia, and between some streptococci and *B. tumefaciens*. The presence of numerous antibacterial substances in the roots, leaves and flowers of many plants would lead us to suppose that the functions and activities of the micro-organisms of decomposition, that is to say, the microbial flora of the surrounding environment, whether cultivated or not, must depend to some extent on the presence of such substances in the soil.

Other activities of micro-organisms

When we look at micro-organisms from the point of view of their part in maintaining the conditions of life on the earth by their powers of breakdown, we find that other functions are of importance, for instance, those involving the circulation of the various substances essential to animal and plant life. The cycles of nitrogen, sulphur, iron and manganese which are maintained by organisms in the soil, are of particular importance.

Both in virgin land and cultivated soil there is a complex microbial life which may be the object of specific microbiological studies of the ground, inasmuch as the biochemical activities of the soil flora are at one and the same time the cause and the effect of those conditions which experts identify in the fertility of the soil, that is to say, of the conditions which make soil suitable for the growth of cultivated plants. The nitrogen cycle, which begins with the breakdown of organic proteins, ends up, as we have already seen, with the production of ammonia, but it also includes other steps of great interest, such as nitrification and the fixation of free nitrogen from the air.

Nitrification and denitrification

By nitrification we mean the transformation of ammonia by a process of oxidation into nitrous acid and nitric acid, successively, both of which then rapidly form salts, in particular with calcium and the other metals present in the soil. This process occurs not only in the soil, but on various materials too. It was first noticed on walls which were particularly damp, and which were in contact with ammonia vapour (in stables, for example). Under these conditions there occurs the formation of saltpetre (sodium nitrate), important in earlier days as one of the constituents of gunpowder. This process of oxidation, first interpreted in its purely chemical aspect by Liebig, was explained by Pasteur as being the result of microbial action. This idea was confirmed by the work of Schloesing and Muentz, while the bacteria responsible were finally isolated in pure culture by Winogradsky.

This phenomenon consists effectively in the gradual oxidation of ammonia by bacteria of the genus *Nitrosomonas;* this leads to the formation of nitrous acid. This is followed by the further oxidation by bacteria of the genus *Nitrobacter*, to nitric acid. These latter micro-organisms are particularly interesting because they obtain the energy necessary for their growth and reproduction from these oxidative reactions, which are exothermic:

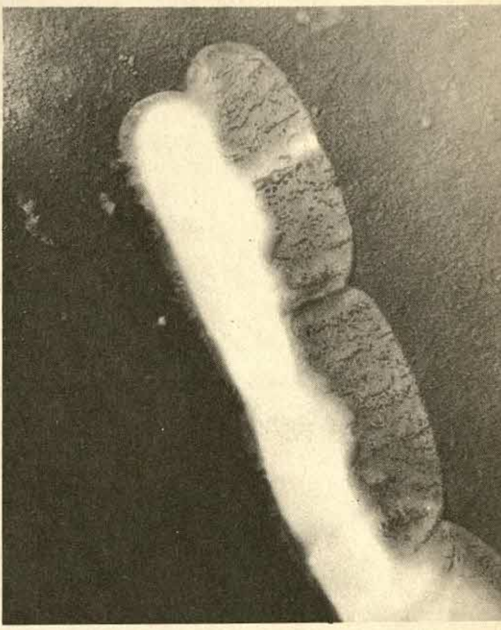
$$NH_3 + 3O \rightarrow HNO_2 + H_2O + 79 \text{ calories}$$
$$HNO_2 + O \rightarrow HNO_3 + 21 \text{ calories,}$$

using simultaneously the carbon of free carbon dioxide for protoplasmic syntheses, as do the green plants, but without the intervention of chlorophyll, which is not present. This activity of bacteria in nitrification leads therefore to the slow but continuous transformation of ammonia derived from the decomposition of materials into nitrites and nitrates, soluble in water and promptly assimilable by plants. This process is of fundamental importance for the life of the plant world, since it ensures the release of nitrogen bound in organic plant and animal substances for the further use of plants. In forest soil, which is often too acid for the growth of the above-mentioned genera, one finds the specific forms belonging to the genera *Nitrosocystis* and *Nitrosospira*, of which the biochemical activities are analagous to those we have mentioned.

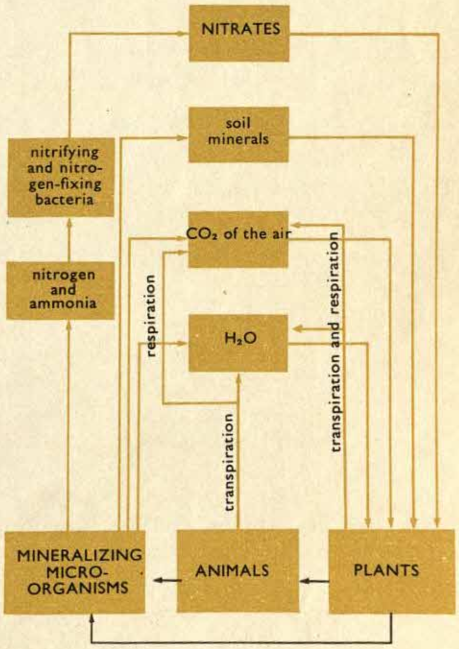
The reverse process, by which nitrate is converted into nitrite and then ammonia, is known as denitrification, and is brought about by the denitrifying organisms. The function of denitrification can be assumed by numerous heterotrophic micro-organisms and by certain micro-organisms which seem to have specific activities (among these we may note the first of the denitrifiers isolated by Gayon and Dupetit in 1886, called *Bacillus denitrificans*). Whereas the process of nitrification is favoured by the presence of air, denitrification proceeds best in the absence of air. It is clear that nitrification is of great interest in connection with the purification of water, and such processes are favoured by the methods of treatment of water from sewage called the 'activated sludge' processes.

Fixation of nitrogen and reduction of sulphur

The fixation of atmospheric nitrogen is the process which follows the intercepting of nitrogen from the air with formation of inorganic or organic nitrogen compounds; finally the nitrogen is put at the disposal of plants. This process is brought about by different micro-organisms, some of which are found living in symbiosis with nodules on the roots of leguminous plants, while others live in natural colonies in the soil. The power of leguminous plants to fix nitrogen is a phenomenon known from ancient times, but the first conjecture related to this function of fixing atmospheric nitrogen was due to the work of Vill and was raised in 1850. The first demonstration of the existence of special bodies, called 'bacteroids' in the roots of leguminous plants was made by Gasparrini in 1851. Finally, in 1888, the actual demonstration of the part played by bacteria in the process of fixation was made by Prazmowski and Beijerinck, who isolated in pure culture an organism from beans which was called *Bacillus radicicola*, now known as *Rhizobium leguminosarum*. The fixing of the free nitrogen of the



This is an electron microscopic view of Bacillus subtilis, *of which the spores are shown on p. 69. The spores, which enable the bacteria to spread, are easily visible and make it appear as if the cell has divided into two. (Mag. × 30,000 I.N.R.A).*


NITRATES
soil minerals
nitrifying and nitrogen-fixing bacteria
CO₂ of the air
nitrogen and ammonia
respiration
H₂O
transpiration and respiration
transpiration
MINERALIZING MICRO-ORGANISMS
ANIMALS
PLANTS

The nitrogen cycle

Continued from page 68

1878
MIGUEL *observes and describes several types of bacteria and cocci which act specifically on urea* (Urobacillus pasteureii *and* Micrococcus ureae).

1877
SCHLOESING *and* MUENTZ *show that nitrification of ammonia is due to bacterial action.*

1888
BEIJERINCK *isolates the bacillus of tubers.*

1890–91
WINOGRADSKY *isolates specific germs from pure culture.*

1893
WINOGRADSKY *demonstrates anaerobic fixation of nitrogen and isolates the specific organism,* Clostridium pastorianum.

1901
BEIJERINCK *shows aerobic fixation of nitrogen by organisms of the genus* Azotobacter *and isolates these organisms in pure culture.*

1902
OMELIANSKI *shows the existence of anaerobic micro-organisms which are capable of breaking down cellulose, and isolates the two principal species,* Bacillus fossicularum *and* Bacillus methanigenes.

1916–17
CARBONE *isolates, describes and conducts experiments with the specific organism,* Clostridium felsineum, *which acts on pectins.*

1919
HUTCHINSON *and* CLAYTON *demonstrate specific aerobic organisms which break down cellulose.*

1929
WINOGRADSKY *takes up the subject again and classifies the organisms of aerobic cellulose decomposition in the genera* Cytophaga, Cellvibrio *and* Cellfalcicula.

air and its transformation into nitrogen compounds in the root nodule is dependent on the symbiosis of the tissues of the plant root and that of the bacteria. In other words, it is only by the action of enzymes produced from the partners in the symbiosis that the fixation of elementary nitrogen can take place, and depending on a mechanism which has not, moreover, been fully elucidated. According to Virtanen, the rhizobium would fix nitrogen by forming hydroxylamine which would combine with the oxaloacetic acid produced by the leguminous plant. Subsequent transformations in this compound would lead to the formation of different amino acids[3].

The fixation of atmospheric nitrogen by free-living organisms was first demonstrated by Winogradsky, who isolated *Clostridium pastorianum*. This is an anaerobic spore-bearing organism which acts in an environment in which air is absent, obtaining its energy by the breakdown of carbohydrates present in the soil that arise from the previous microbial breakdown of cellulose. The fixation of nitrogen by this Clostridium has been calculated to amount to 1·4 parts of nitrogen to every thousand parts of carbohydrate used. In the aerobic fixation of nitrogen by organisms of the genus *Azotobacter*, found in most soils, something like ten parts of nitrogen are fixed for every thousand parts of carbohydrates used. In each case the nitrogen is turned into nitrates which are available for absorption by plants.

The cycle of sulphur in the soil comprises both reduction and oxidation. The course of the putrefaction during which H_2S separates itself from molecules of albumin containing sulphur is owing to micro-organisms which decompose organic substances and as a general rule do not present a specific action. On the contrary, this specificity is found for the reduction of sulphites, hyposulphites and particularly sulphates, due mainly to the action of micro-organisms connected with the species *Vibrio desulfuricans*. This latter organism has a great resistance to high concentrations of sodium chloride so that it can act in sea water; it has often been found in salt water rich in organic material due to the decomposition of seaweeds.

The reactions involved in the oxidation of sulphur are the work of the green sulphur bacteria, which use for their synthesis the energy which is liberated during the exothermic reaction transforming hydrogen sulphide into sulphuric acid. These reactions are found in the media in which H_2S is present. The commonest of them are the *Beggiatoae* and the *Thiothrix*. It is not rare to find both sulphur-reducing and sulphur-oxidizing bacteria in the same place. The first occur in the deeper layers away from the air and the second near the surface where oxygen is available.

The cycle of iron and manganese

Among the numerous organisms which are capable of changing insoluble salts of iron and manganese into soluble ones, there are certain ones, called iron bacteria, which have this property particularly well-developed and which work in a characteristic way. Typically they are filamentous, like *Leptothrix ochracea*, *Clonothrix fusca* and those of the family *Gallionellaceae*. These grow with a gelatinous coating almost entirely composed of either iron or manganese oxides. Besides the filamentous forms there are also coccoid and bacillary ones which themselves determine an effect of precipitation on the salts of iron and manganese. The energy necessary for their metabolic activities comes from the exothermic reactions involved in the oxidation of various iron compounds. For example:

$$2FeCO_3 + 3H_2O + O \rightarrow Fe_2(OH)_6 + CO_2 + 29 \text{ calories.}$$

It should be noted, however, that in certain cases the fixation of iron and manganese, as for the compounds of phosphorus, potassium and calcium, is related to the activity of numerous micro-organisms, schizomycetae and eumycetae that may have no specific action on these substances, but produce their effects by forming acids to the extent that the solubilization of iron and manganese follows the cycle of other minerals.

In conclusion, the microbial processes which take place in the soil are stimulated or limited by numerous factors, some physico-chemical, others typically biological. The predominance of one species, which may be determined by the availability of food, or the temperature, can bring about a physico-chemical change sufficient either to stimulate and be favourable to the life of other microbial species having different action upon the organic substances present, or, on the contrary, to limit it. The bacterial equilibrium of the soil, and therefore the maintenance of life, is the result of the interplay of many factors in ways as yet poorly understood and little investigated experimentally, factors which indirectly govern and direct the activities of micro-organisms relative to mineralization and to synthesis.

Marine microbiology

Holger W. JANNASCH

Marine microbiology is the study of the biological, chemical and geographical problems connected with the existence of micro-organisms in the sea. The importance of such a study is evident, for it is the infinitely varied metabolic[1] *activities of micro-organisms which lay the foundation for the majority of the large-scale processes which develop on the earth's surface. The sea not only covers the greater part of this surface but also makes up the greater part of the total biosphere.*[2] *Because a portion of the organic matter from the soil is constantly carried away towards it, the sea has been said to be the biggest septic tank in the world.*

The marine flora can be considered either in terms of its surface distribution (horizontal distribution) or in terms of its distribution in depth. In the first case, two factors play an important part in the qualitative and quantitative distribution of the microbial flora. The first is the instability of the oceanic mass, an instability caused by winds, tides, currents, seasonal influences, the degree of solar heat, the concentration of salt in the water, and the diurnal changes in the environment. The second is the distance from the coasts and the influence of discharge from river estuaries, especially those near centres of population.

Distribution in depth, or vertical distribution, is concerned with the following factors: pressure, temperature, biological factors (as far as the microbial marine flora is closely linked with the plant and animal populations of the environment), and finally the speed of sedimentation of organic material (as far as this material constitutes a centre of microbial multiplication).

It follows therefore that, in addition to the specific and permanent marine flora, there is also to be found in certain intermediate zones like coastal waters, estuaries and shallows, an 'accidental' flora which can survive in the sea for only a very short time and which is much more like the microbial population of the soil and fresh water. There also exists an 'ubiquitous' flora which can adapt itself equally well to marine conditions and to terrestrial ones.

But if we limit our study to the specific and permanent marine flora, this can be seen to fall into three groups: the surface flora, which is found in zones of only a few yards in depth and which reflects all the various changes in the surface of the sea; the pelagic flora, which is spread out between the surface and the benthic flora, is very limited in quantity, and is subject to the ocean currents and the sedimentation of organic matter which these carry with them; and the benthic flora, which is characteristic of the sea bed and is more stable, since it is much less influenced by changes in environment.

The conditions of life of the marine micro-organisms

One of the chief characteristics of the marine environment is the homogeneous distribution of the saline matter which is dissolved, as well as the extremely constant chemical composition of sea-water. On the other hand, those constituents which are absorbed by micro-organisms in sea-water are found in variable concentrations; this refers particularly to the compounds of carbon, nitrogen and phosphorus.

In conformity with 'Liebig's law' these differences in concentration condition the existence and the activity of marine micro-organisms. Otherwise their growth is scarcely

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(1) Metabolic reactions: *metabolic reactions comprise the overall changes which living organisms undergo, except for those directly connected with nutrition. One can distinguish three types of microbial metabolism: energy can be obtained by* (a) *the assimilation of organic substances (heterotrophic micro-organisms)*; (b) *the transformation of inorganic substances (chemotrophic micro-organisms): and* (c) *the use of light energy (phototrophic micro-organisms).*

(2) Biosphere: *the region in which physical and chemical conditions are suitable for life.*

(3) *It seems nevertheless that low temperatures in the sea depths do effect a selection among the various species of micro-organism (see Zobell, and also* 'The Animal World', *p. 37).*

(4) *To show the presence of bacteria in sea water a small sample is added to a nutritive medium and after a certain period of incubation the number of bacterial colonies is counted. (Koch's plating-out method).*

(5) Heterotrophic *micro-organisms. See Note 1. The natural population of the sea is made up equally of chemotrophic and autotrophic organisms which do not develop in this method of culture.*[4] *This method reveals mostly the presence of Gram-negative bacteria (95 per cent); a few cocci, some spore-bearing bacteria, and a few Gram-positive bacteria are also found.*

affected by such factors as the concentration of hydrogen ions (subject to only very slight changes), or temperature differences which have no direct action on the metabolism of the micro-organisms.[3] As for pressure of water, which is very great in the depths, this does not appear to upset the microbial activity; at the most it exerts a selecting influence among the various microbial species.

The presence of micro-organisms, especially bacteria, in every drop of sea water, was demonstrated a very long time ago. The surface waters of the broad expanses of tropic seas, which are poor in nutritive substances, have been shown by cultural methods[4] to contain less than 10 bacteria per millilitre. In the rich regions of the Sargasso sea this number rises to 1,000 per millilitre; but in the coastal regions, and on the sea-bed counts of from 100,000 to several million organisms per millilitre have been obtained. If, instead of using cultural methods, we look at sea water directly through the microscope, we may get figures up to 3^{10} higher than these, seeing that the nutritive media used in culture allow the growth of only part of the heterotrophic[5] micro-organisms present. But the direct counting of organisms under the microscope presents fairly considerable technical difficulties, and gives us no information about their metabolic activities. At the present time there is a tendency for methods which measure metabolic activities to replace the counting of organisms.

Certain groups of marine bacteria, yeasts and fungi are adapted to narrowly specific conditions of life. Such are the 'heliophilic', 'barophilic' or 'psychrophilic' species. Other groups, on the other hand, are scarcely distinguishable from those found in the soil or in fresh water, for example, those species which are called 'halotolerant'.[6] One also finds in the sea Actinomycites, such as Streptomyces,[7] yeasts, and numerous fungi which are specifically marine, as parasites on plants or ocean creatures. We have, however, little information on the part such organisms play in the general metabolic activity of the marine microbial flora.

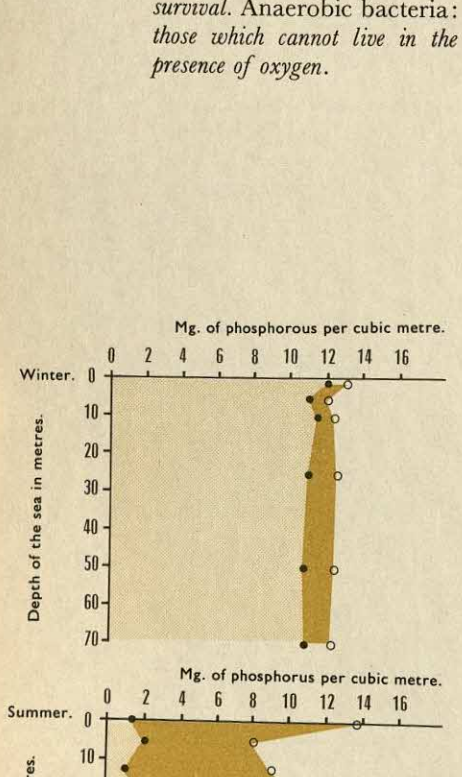
In the surface region of the sea reached by the sunlight, microscopic plants are dominant. These undergo photosynthesis, assimilate the nutritive substances, and then fall gradually into the deeper layers and are decomposed by bacterial action during their fall. Thus the nutritive substances set free by decomposition accumulate in the depths of the sea. This is why, in spite of the abundance of light and a higher temperature, the surface region tends to become poorer in life, as shown by its transparency and deep blue colour. As for the water in the depths, rich in nutritive substances, it drifts either northwards or southwards, following the deep currents, and then, when it comes into contact with the cold water of the polar regions, it rises to the surface. In these regions the green colour of the sea indicates its abundance of planktonic life.

The breaking-down processes of the micro-organisms being of an oxidizing nature, the concentration of oxygen in the water is a measure of their intense activity. However, unlike what occurs in inland waters, it is rare for marine oxygen to be entirely used up through the breaking-down activity of bacteria. This happens in only a few regions, such as the deeper layers of the Black Sea, certain Norwegian fiords, and those tropical seas that are more or less closed. Oxygen is equally scarce in the upper layers of the sea-bed where the sedimentary organic substances accumulate. In such regions one finds anaerobic bacteria[8] whose action reduces sulphates, nitrates and carbon dioxide to produce hydrogen sulphide, free nitrogen and methane.

Marine micro-organisms and the cycles of carbon, nitrogen and phosphorus

All micro-organisms liberate, in the form of carbon dioxide, the carbon of complex animal and plant constituents (sugars, starch, cellulose, proteins, fats, urea, chitin, etc). Ninety per cent of the carbon dioxide of the biosphere thus comes from the metabolic activity of plants and of micro-organisms. In this way, by transforming part of the organic substance into cellular matter, the marine micro-organisms render it capable of feeding the small animals which are found in the sea and which filter the substances.

Only certain very specific micro-organisms take part in the metabolism of carbon and phosphorus. In general, these organisms can use only bound nitrogen. This is present in nature in only very slight quantities, about 0·0001 per cent of the total nitrogen. Only a few groups of bacteria, called denitrifying bacteria, can catalyze the change from free nitrogen to bound nitrogen, in the same way as blue algae. In the metabolism of bound nitrogen there are marine chemotrophic bacteria which oxidize ammonia into nitrate, the ammonia having been the final product in the



Differences in the concentration of phosphorus at various depths during summer and winter.

[6] Heliophilic: *organisms adapted to exposure to sunlight.* Barophilic: *organisms adapted to life under high hydrostatic pressure.* Psychrophilic: *organisms which can live at low temperatures.* Halophilic: *organisms which are adapted to life at high salt concentrations.* Halotolerant: *organisms which can live at both low and high salt concentrations.*

[7] Streptomyces: *micro-organisms which produce antibiotics; their presence and activity in sea water has so far been little studied.*

[8] Aerobic bacteria: *those which need oxygen for their survival.* Anaerobic bacteria: *those which cannot live in the presence of oxygen.*

Diatoms, brown microscopic algae, constitute the major part of the mass of floating micro-organisms of which the whole group makes up the plankton. (This is the species Navicula, magnified 5,000 times). The single cell of a diatom has a siliceous shell made up of two parts which fit together. (Photo Lod).

breakdown of organic substance; the nitrate thus formed serves as food for plants.

The metabolism of phosphorus is less complicated, since phosphorus is always combined in the form of phosphate, in organic as well as inorganic compounds. Phosphorus is not only one of the constituents of the cell; it also forms a transport system for energy, either combined with iron or accumulated as a reserve in the organisms.

The metabolism of sulphur is similar to that of nitrogen, and again, only micro-organisms take part. Wherever one of the constituents is found in abundance, for example, sulphate or hydrogen sulphide, a characteristic development of the masses of micro-organisms is seen. In estuaries, zones rich in sulphate, the anaerobic sulphur-reducing bacteria produce hydrogen sulphide. This is reoxidized at first by the aerobic bacteria which are found where the oxygen-free layers of water are in contact with those containing oxygen, and elementary sulphur is produced. The cycle is then continued by the anaerobic photosynthetic bacteria which are often responsible for the red colour that is characteristic of water containing hydrogen sulphide. The free sulphur can be reoxidized to sulphate, thus completing the cycle. The micro-organisms which take part are halophilic and develop in masses, even when continual evaporation has raised the salt concentration of the water to saturation point.

Marine microbiology has numerous practical aspects and applications. It is useful to the naval architect, who has a constant struggle against the flora which fouls the hulls of ships, and to those concerned with the fishing industry and sewage disposal. The protein matter of fish roe, very susceptible to bacterial decomposition, can be preserved in a fresh state only by the application of microbiological knowledge and techniques. Oysters and mussels are often carriers of pathogenic bacteria and special methods are needed for their purification. The lethal action of sea water on intestinal bacteria is very important in sewage disposal; part of this action has been attributed to substances analogous to antibiotics, but the accuracy of this theory this has not yet been established.

The microbiology of oil

Serghiei I. KOUZNETSOV

Of all the branches of bacteriology, the microbiology of oil is the most mysterious. In the narrow sense of the term, this branch of science is the study of the action of a certain microbial flora which is capable of determining the formation, maturation, movement, and accumulation of oil. The distribution of this flora, both in depth and on the surface, gives us many helpful indications in the search for oil, and for its extraction. In the microbiology of oil can also be included micro-paleontology, which is the study of the influence of micro-organisms upon geological deposits, and which enlightens us on the mechanism of oil formation, as well as the study of the micro-organisms which attack the structure of the oil-wells, and of those which can break down hydrocarbons and thus give rise to many derivatives of oil: but this touches on the industrial aspects of microbiology and we shall not deal with these here.

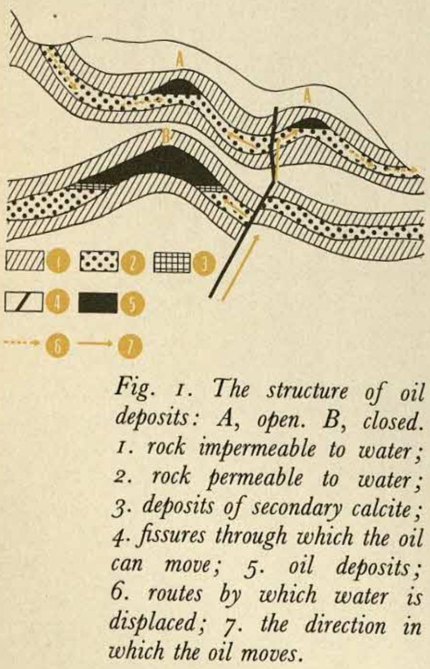


Fig. 1. The structure of oil deposits: A, open. B, closed. 1. rock impermeable to water; 2. rock permeable to water; 3. deposits of secondary calcite; 4. fissures through which the oil can move; 5. oil deposits; 6. routes by which water is displaced; 7. the direction in which the oil moves.

At present there are two theories on the origin of oil: the 'mineral' theory and the 'organic' theory. Porfiriev, basing his views on the researches of Hoyle, Simon, Gold, Süss and others, suggests that oil as we find it is a substance as elementary as iron, methane, water and the minerals which constituted the nebulous protoplanetary matter from which the earth and the other planets were formed. At the time of the repartition of this substance, there was formed a system in which siliceous rocks would provide the 'skeleton' and the clefts would have been filled with molten iron and with the lighter fluids, water, and the hydrocarbons of oil and gas. The liquid iron phase would have been denser than the rocks, which it would therefore penetrate to form the central part of the earth; the lighter fluids would be nearer the earth's crust. In the course of the gigantic foldings of the earth's crust, during the proterozoic and tertiary eras, the water and the hydrocarbons would have come to the surface through breaks, the water forming the seas and the hydrocarbons the oil deposits.

This 'mineral' theory is supported by a variety of geological facts, in particular by the frequent presence of oil in crevasses in the crystalline lower layers of rocks, and by the fact that oil deposits always occur in regions where cracks in surface rocks are common.

According to the theory of Goubkin, on

the other hand (the organic theory), organic matter, under anaerobic conditions,[1] would fill the muddy sediments of the sea-bed polluted by the hydrogen sulphide arising from fermentation. Thus the parent material of the oil deposits would be formed. In these deposits organic matter would be further transformed by biological or chemical action, and under the high pressure of the overlying rocks the oil would be squeezed out of the rocks in which it was originally present and be gradually drained through fissures in the surrounding rocks to form deposits. If oil has been formed from organic matter, bacteria indubitably have played a part in its formation.

The role of bacteria according to the organic theory

The remarkable work of Zobell suggests that the process of oil formation would take place in the following manner. Under anaerobic conditions the bacteria would have decomposed the plant and animal remains with the formation of methane and fatty acids. At the same time would have been released a small quantity of liquid hydrocarbons, a constituent part of the plant cell. The hydrocarbons resulting from the reduction of the fatty acids would have accumulated in the bacterial cells. These hydrocarbons were present in only a small quantity to begin with, but being more resistant than the protoplasm of the bacterial cell, they would be able to accumulate in considerable quantities. It is thus that oil would have accumulated in the oil-bearing rocks.

By the action of other bacteria capable of activating molecular hydrogen, the unsaturated hydrocarbons would have become saturated. Eventually, the bacteria, in forming carbon dioxide or methane, would have saturated the oil of these gases whilst increasing its mobility, and would have contributed to the accumulation of the oil in the storage rocks.

It has been possible to show empirically the exactitude of each of the stages in the process of decomposition of plant residues by bacteria, but, as a whole, the organic origin of oil is up to now only a theory which is unsupported by fact.

The repartition and activity of bacteria in oil deposits

Deposits of oil and gas accumulate in anticlinal folds covered by slate or other types of rock impermeable to water. The oil is generally in contact below with infiltrating waters which shift equally in the direction of a layer or horizon[2] impermeable to water. The contact between the layers of oil and water may be more or less complete, or, on the other hand, entirely non-existent. In the latter case the deposit is said to be 'sealed'. As a rule such a separation between oil and water is due to the formation of a thin layer of calcite, called secondary, at the interface (fig. 1). This deposit of secondary calcite is produced by the activity of sulphur bacteria which reduces the calcium sulphate frequently present in the infiltrating water. The quantity of micro-organisms present gives a good indication of their activity, which depends to a considerable measure on the extent of the water exchange in the deposit.

The number of micro-organisms present in a deposit increases considerably after a few years of pumping. For example, Sturm showed that after eight years of exploitation of the deposits at Syrzan, the number of micro-organisms had risen from 50,000 per millilitre to several hundred thousand, or in an extreme case to seven million. The quantity of bacteria increases considerably wherever the waters within the rocks are displaced and are in contact with the oil. The analysis of plutonic rocks by Ekzertsev has shown that bacteria are found particularly in productive horizons and may amount to several millions per gram of rock.

Bastine was among the first to show the presence of sulphate-reducing bacteria in the infiltrating waters. It appears that before the hydrocarbons can be utilized by the desulphurizing bacteria, the oil must be decomposed to some extent by other species. The number of such bacteria in infiltrating water may amount to a million per millilitre and the presence of great quantities of sulphites and of hydrogen sulphide is a result of the reduction of sulphates by these organisms.

The activities of sulphur bacteria are seen even more markedly in deposits in which either fresh or salt water is pumped into the rocks to aid a second extraction of oil. In a series of such deposits in the middle Volga over a period of four months an increase in the quantity of hydrogen sulphide was observed. This quantity in some cases amounted to 120 mg/l (1 mg/l per 24 hours). In a deposit at Chor-Sou in Central Asia, Ivanov proved by the use of radio-isotopes that this hydrogen sulphide was formed biologically, reaching 0·2 mg/l in twenty-four hours.

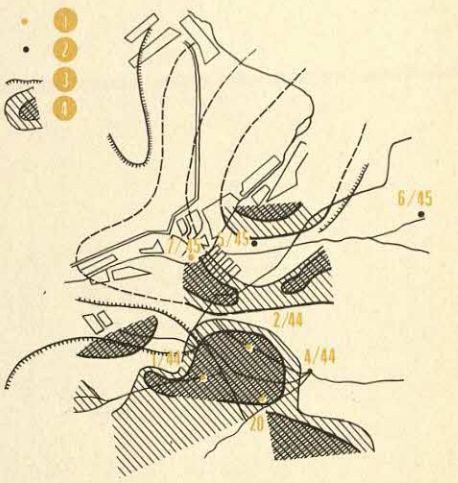


Fig. 2. The microbiological method for oil prospecting consists in taking samples of the water or rocks being investigated in order to study the distribution of the microbial flora which gives rise to hydrocarbons. 1. a boring where gas is present; 2. a boring where there is no gas; 3. limit of the distribution of the bacteria concerned with hydrocarbons in the soil water; 4. zones marking the density of the microbial population as it varies over the area covered by the samples.

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(1) Anaerobic conditions *are those where oxygen is absent;* aerobic conditions, *where oxygen is present.*

(2) Horizon: *in geology, a horizontal layer in a section of the rocks.*

HISTORICAL NOTES

1895
MIYOSHI *observed the decomposition of paraffins by fungi and moulds.*

1901
SHEIKO *discovered bacteria in oil.*

1908
STORMER *proved that bacteria can use aromatic hydrocarbons.*

1926
BASTINE & GUINSBOURG-KARAGUITCHOVA *discovered simultaneously and independently that the oil-bearing rocks contain sulphate-reducing bacteria. They postulated that the hydrogen sulphide found in oil deposits is formed by bacterial action.*

1928
TAUSSON *proved that crude oil can be oxidized by bacteria.*

1937
MOGUILEVSKI *took out a patent for the microbiological method of prospecting for oil.*

1943
ZOBELL *put forward his idea of the role of bacteria in the geological process of oil formation.*

1950
STURM, *using the direct microscopic method, showed that the number of bacteria in infiltrating water may vary between tens of thousands and millions per millilitre.*

1956
EKZERTSEV *showed that under anaerobic conditions oil may be decomposed by bacterial action with the formation of combustible gases.*

The fact that one always finds gaseous hydrocarbons in oil deposits suggests that the formation of methane and other gases is due to the decomposition of the oil under anaerobic conditions. With this in mind Ekzertsev and Kouznetsov studied, in a number of deposits containing oil and gas, the distribution of the methane-producing bacteria in the waters of the oil-bearing rocks. Analysis showed that in almost all the deposits bacteria could be found which were capable of forming methane from hydrogen and carbon dioxide, and which could break down fatty acids to form methane. In many cases it was possible to detect the formation of gaseous products if infiltration water was added to Barker's medium, in which the sole source of carbon was oil.

This fact has allowed the assumption of the presence, in oil deposits, of micro-organisms which break down the oil with the formation of gaseous products.

Ekzertsov described about twenty strains of bacteria which can be shown, in the laboratory, to produce gas in an inorganic medium to which sterile oil had been added as the sole source of organic material. Among the micro-organisms described are species closely resembling *Methanococcus mazei* and *Sarcina methanica.* It is interesting to note that the gas dissolved in the infiltration water of numerous oil deposits resembles in its composition that which is formed in laboratory experiments. However, the mechanism, properly speaking, of the process of anaerobic decomposition of the constituents of oil to form gaseous products has not yet been elucidated.

The oxidation of oil and hydrocarbons

The oxidation-reduction potential in oil deposits does not in general exceed $rH_2 = 7$–8, indicating that under natural conditions oil exists in a reduced condition.

As a result of earth movement and high pressure, oil can sometimes come up through the fissures until it is exposed to the air. Then, as a result of the lowering of the pressure and the temperature, the various constituents can divide to give deposits of paraffins, ozokerite and various bituminous tars. At the same time some of the constituents of the oil may volatilize or may be oxidized by bacterial action. Petrol from great depths contains few bacteria and those which are found are mainly capable of oxidizing the heavy fraction. In the rocks which contain ozokerite there are no bacteria capable of oxidizing hydrocarbons; but if ozokerite is placed in an oxidizing environment it may be attacked by such bacteria.

Tausson has studied the oxidation of oil which has reached the air through natural processes. He succeeded in isolating a large number of bacterial species capable of oxidizing the individual constituents. They include *Bact. aliphaticum liquefaciens*, *Bact. hydrium*, *Bact. toluolicum*, *Mycobact. album*, *Mycobact. rubrum*, etc. Tausson estimates the rate of oil oxidization on the earth's surface at twenty-five tons per 2½ acres (approximately) a year.

The oxidation of combustible gases

Gases accumulate in practically all oil deposits. When the pressure in the rock strata reaches several hundred atmospheres a large quantity of gas dissolves in the oil and in the infiltrating water. In proportion as oil is withdrawn from the deposit, the pressure in the strata is lowered, the solubility of the gases in the oil is reduced, and the least soluble gases like nitrogen, methane, ethane and propane are the first to escape. By the time that the pressure has reached that of the atmosphere, a maximum of about 325 cubic metres of gas may have been released from a ton of oil (this figure is for deposits in Devonian rocks). The petroleum gases which are under high pressure come up through fissures and through tectonic crevasses as well as by diffusion through the sedimentary rocks into regions where the pressure is less. It is here in the superficial layers that biological oxidation of the hydrocarbons occurs.

Many micro-organisms can take part in the oxidation of methane, ethane and propane. They are: *Bact. methanicum*, *Mycobact. methanicum*, *Mycobact. flavum var. methanica*, *Mycobact. perrugosum var. ethanica*, *Mycobact. paraffinicum*, *Mycobact. rubrum var. propanica*, *Nocardia sp.*, *Pseudomonas propanica*, etc. Many of these micro-organisms oxidize certain hydrocarbons selectively.

The concentration of gaseous hydrocarbons in the rocky strata seldom rises above 0·001 per cent, even immediately above oil deposits. Nevertheless, even at very low concentrations, the normal development of bacteria which oxidize methane, ethane and propane, is possible. It is by studying the distribution of such specific organisms that it has been possible to develop microbiological methods of prospecting for oil.

Bacterial exotoxins

William E. Van HEYNINGEN

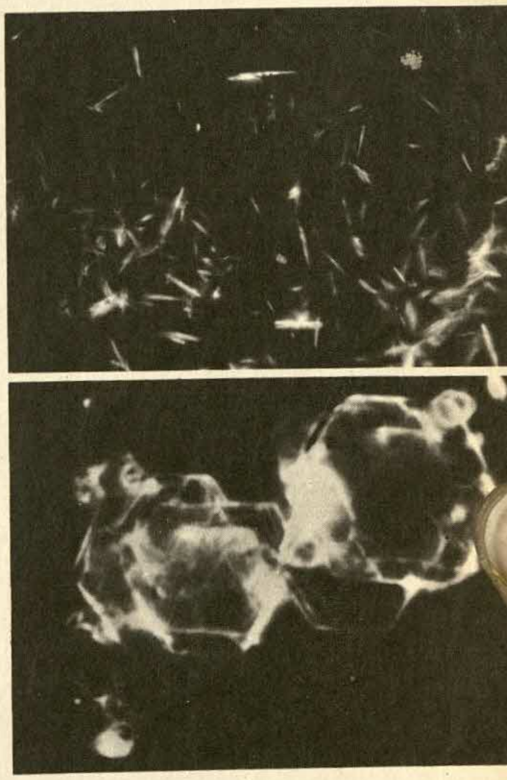
Some germs (tetanus, botulinus, diphtheria, staphylococcus, etc) make and spread around them specific poisons that are called exotoxins because of the way in which they are produced. These poisons spread within the infected organisms, where they can damage tissues and body-fluids, making life impossible. In this case we can say that death results less from an infection than from a poisoning.

The invasion by pathogenic bacteria of the tissues of a host may account for the setting-up of an infectious disease,[1] but it does not explain how the harmful effects of the disease are brought about. The total weight of the invading parasites is not more than a few grams at most, and generally less. The mere presence, *per se* of such a small amount of foreign matter in the body is not harmful, and other reasons for its obnoxiousness must be found. A partial explanation lies in the poisons, of greater or less potency, produced in greater or less amounts by most pathogenic bacteria. In a few cases such poisons account entirely for the harmful effects of the disease; in most cases poisons are produced, but we are not sure what part they play. These poisons are known as bacterial toxins.

Exotoxins and endotoxins

There are exotoxins and endotoxins. The prefixes *exo-* and *endo-* imply that the toxins respectively are secreted by the bacterial cell and exist outside it in the culture medium, or exist within the cell as part of its substance. But the exotoxins are often found within the cell and endotoxins outside, and the prefixes are now retained only out of habit and convenience.

The difference between the two is more fundamental. The exotoxins are proteins,[2] and therefore large molecules. Being proteins, they are unstable to heat, and they are antigenic, that is to say, when they are introduced into the animal body they stimulate the formation of antibodies, and these antibodies neutralize the toxic activity. They are capable of being converted, naturally or artificially, into anatoxins, or toxoids, that is, of being rendered non-toxic without losing their ability to stimulate the formation of toxin-neutralizing antibodies. Each exotoxin is a different protein, with its characteristic biological activity, and, as we shall see, the biological activity of these large-molecular proteins is much greater than that of small-molecular substances. The endotoxins are even larger molecules than the exotoxins, consisting of complexes of protein, lipid[3] and polysaccharide,[4] but the toxic component of these complexes is a small molecule, a lipid, which we may look upon as a 'passenger' within the larger complex. The complex is heat-stable and antigenic, but the antibodies are orientated towards the protein and polysaccharide components and they do not neutralize the small toxic component. The small-molecular toxic components of all endotoxins are the same, irrespective of the parent organism, and therefore they all have the same biological activity, an activity which is much



The two protein components of staphylococcal leucocidin. These destroy white cells, but are not active separately; together, they constitute active leucocidin. (After Woodin, 1960).

lower than that of the biologically active proteins.

The exotoxic proteins occur in a number of different forms. Some are produced by the bacterium as inactive protoxins, which are converted into active toxins by enzymes in the culture medium. In some cases (eg tetanus toxin) the molecules of a toxin may associate to form larger molecules; conversely, the large molecule of botulinus toxin may dissociate into smaller protein molecules, some of which appear to be toxic and others non-toxic. At least three exotoxins, plague toxin, anthrax toxin and staphylococcal leucocidin, appear to consist of two different protein components. These components are not toxic when separated, but together they constitute the active toxin. But despite these various manifestations the exotoxins remain simple proteins, simple in so far as they consist only of polymers[5] of amino acids, without any other chemical groups attached.

Formidable poisons

The toxicity of the bacterial exotoxins is appalling. It will be seen that the exotoxins listed in the table (p. 84) are hundreds or thousands of times more potent than such well-known small-molecular poisons as strychnine or arsenic, or the most powerful poisons of snake venoms, poisonous fishes or plants, or the bacterial endotoxins. This table needs some explanation. It is difficult to make comparisons of toxicity because the species of experimental animals upon which toxicity tests are made vary in weight as well as susceptibility to a considerable degree. Consequently it might be better, objectively, to list the experimental animals and the smallest doses which kill them, and to refrain from making comparisons; but subjectively we are interested in man, and interested in making comparisons. Therefore, the lethal doses for man have been calculated on the basis of two assumptions: (a) that the size of the lethal dose increases with the weight of the individual, (b) that man is as susceptible, weight for weight, as the most susceptible animal that has been tested. The first assumption is more justifiable for some poisons than for others; the second assumption, for obvious reasons, cannot be tested.

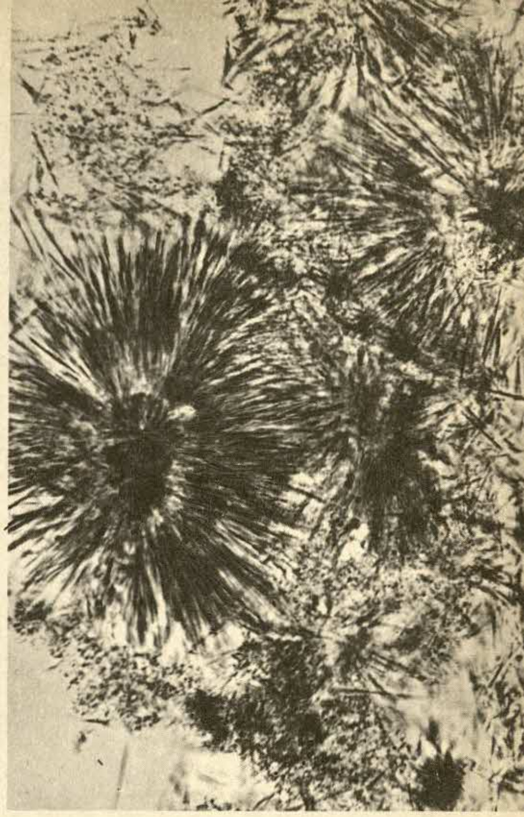
It must also be noted that the lethal doses shown in the table are administered by injection directly into the blood stream or the tissues; by other routes, such as the mouth, nose, lungs or skin, the exotoxins would be 50,000 to 1,000,000 times less toxic (owing to poor absorption of the large molecules), whereas the toxicity of the small-molecular poisons would not be much affected. By these routes, therefore, the great differences in potency between the exotoxins and strychnine are not apparent.

The potency of the neurotoxins[6] (dysentery, tetanus, botulinus) is such that we can make an *entirely theoretical* calculation that one milligram would be the lethal dose for a million kilograms of living matter, or that 100 grams would be enough to wipe out the entire human population of the world. It was reported some years ago from South Africa that under certain circumstances the virulence of botulinus toxin could be increased to such an extent that the lethal dose for a mouse was 0·000,000,000,001,500 mg; or 10 mg (the weight of the ash one might flick from a cigarette) for the entire human population of the world. This report has not yet been confirmed, and must therefore be treated with reserve, but it must not be ignored. It was made by a group (since disbanded) of responsible, experienced and competent research workers, and such considerations are not to be ignored.

The exotoxins listed in the table are the most potent, most important, and best known, but there are at least fifty more besides these. Apart from their lethal effects on the various tissues, organs and fluids of the body, many exotoxins are histotoxic, that is, they destroy the cells in the tissues of the body. The collagenase toxins break down the scaffolding material of muscular and other tissues; the hyaluronidases, or spreading factors, dissolve the cementing substance of tissues; scarlet fever toxin injected into the skin of a susceptible subject produces a red flush (0·000,000,000,500 mg is enough); the enterotoxins act on the gut and cause nausea and vomiting; the haemolysins destroy the red blood cells; the leucocidins destroy the white blood cells; the fibrinolysins prevent the blood plasma from clotting when it should; and the coagulases cause the plasma to coagulate when it should not.

How these substances work

Of what use are these poisons to the bacteria? In some cases we know. For example, many exotoxins, such as the α-toxins of *Clostridium perfringens* or the diphtheria toxins, are essential for the growth of the organism in the animal body because they kill the tissues, and the organism is unable to grow in living tissues. The collagenases and



Crystals of diphtheria toxin.

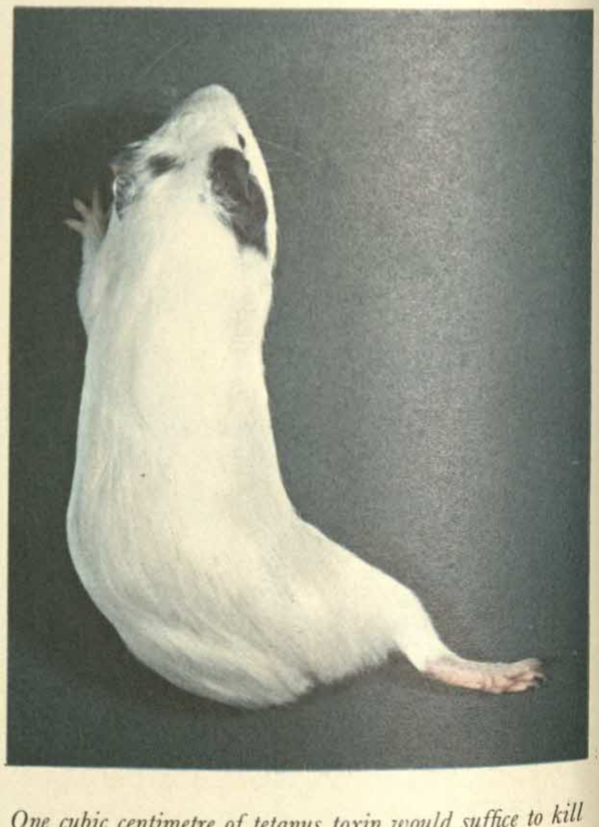
(1) Infectious disease. *A disease caused by parasites, such as bacteria, viruses, protozoa or fungi, as distinct from a degenerative disease.* Pathogenic. *Giving rise to disease.*

(2) Proteins. *Large molecules composed essentially of several hundreds of various amino acids joined together, the basic amino group of one acid being coupled with the acidic group of the next in the chain.*

(3) Lipid. *A small-molecular fatty substance.*

(4) Polysaccharide. *A large-molecular sugary substance.*

Some bacterial cultures assume strange forms (opposite page): here, branched colonies of Bacillus mycoides have been obtained on a peptone gel medium. At the top of the picture is to be noted a quite different yellow colony, that of golden staphylococci.



One cubic centimetre of tetanus toxin would suffice to kill 250,000 guinea-pigs. The effect, as seen here, is spectacular: injected some hours previously with an extremely low dose, these guinea-pigs show the violent muscle contractions that precede death. (Photo Lod. Institut Pasteur).

hyaluronidases aid the spread of bacteria by breaking down the structural components of tissues that stand in their way, and so forth. But such arguments do not apply to botulinus and tetanus toxins. The botulinus bacillus does not even invade the body, and botulism is not an infectious disease, but a food poisoning resulting from the consumption of dead plant or animal matter on which the bacillus has grown and produced toxin. In any case the toxin is not known to bring about any destruction or dissolution of tissues or tissue constituents. The same is true of tetanus toxin, and the tetanus bacillus hardly requires any assistance in its invasion of the animal body. Often it enters through wounds which are so insignificant as to evade detection, and its growth in the body is limited to an extremely small site.

What is the mode of action of these exotoxins? Despite their enormous activity, and despite the fact that some of the toxins (especially the more potent ones) have been known since the last century, we have only a poor understanding of this problem. To understand the mode of action of a drug or poison we must know the fundamental biochemical reaction that it brings about. We must identify the substance in the body with which it reacts, and we must determine the nature of this interaction. Of the exotoxins listed in the table, only the α-toxin of *Clostridium perfringens* is understood in this way. This toxin is an enzyme, a lecithinase, which breaks down lecithin. Lecithin is an important and widespread constituent of body tissues, and its destruction leads to far-reaching pathological consequences. Diphtheria toxin causes a cessation of protein synthesis in body cells, but we do not know directly how this is brought about. The most challenging problem is the mode of action of the three most potent toxins —the dysentery, botulinus and tetanus neurotoxins. Dysentery toxin does not act directly on the nervous system, but appears specifically to destroy the small blood vessels in the central nervous system.[7] As a secondary result of this vascular destruction the nerve cells degenerate, the nervous system is unable to transmit stimuli to the muscles, and the dysentery-intoxicated animal falls into a state of flaccid, or limp, paralysis. But we have no idea how dysentery toxin causes the blood-vessel damage which starts this train of events.

Botulinus toxin also causes a flaccid paralysis, but by an entirely different mechanism. It attacks the peripheral nervous system[8] at the junctions of the motor nerves[9] and the muscles which they activate. Excitatory impulses originating in the central nervous system are conveyed to the ends of the peripheral motor nerves by means of electrical discharges; a chemical transmitter (acetylcholine) then flows from the motor-nerve ending on to the muscle. Botulinus toxin prevents this chemical transmitter from leaving the nerve ending. Consequently the botulinus-intoxicated animal is unable to transmit stimuli to move its muscles and falls into a state of flaccid paralysis. But we do not understand the mechanism by which the toxin interferes with the passage of the transmitter.

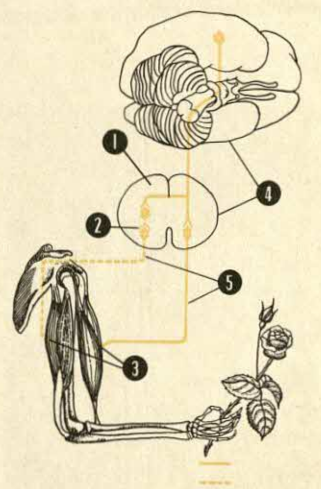
Tetanus toxin also causes paralysis, but it is spastic or rigid, rather than flaccid or limp. The toxin acts on the inhibitory synapses[10] of the central nervous system. Roughly speaking, we might say that every muscle in the body is opposed by another muscle pulling in the opposite direction. Take the example in the accompanying sketch: the forearm is pulled up by the biceps muscle and pulled back to its original position by the triceps. When the biceps receives from the central nervous system via the peripheral motor nerve an excitatory signal to pull, the triceps must at the same time receive an inhibitory signal to restrain it from pulling in the opposite direction. Excitatory and inhibitory signals are passed to the appropriate motor nerves across synapses in the central nervous system. The synapses are junctions between nerves, and, as with the neuromuscular junction, the excitatory and inhibitory signals are carried across them by chemical transmitters. Tetanus toxin prevents the transmitter from acting across the inhibitory synapse. Consequently both sets of muscles pull simultaneously in opposite directions and the jaws, limbs, breathing muscles, etc, of the tetanus-intoxicated animal are rigidly locked in a state of spastic paralysis. Again we do not understand how tetanus toxin prevents the passage of the inhibitory transmitter, but at least the possibility has recently arisen that the substance with which the toxin combines in the central nervous system has been identified.

Exotoxins and disease

What part do exotoxins play in disease? Diphtheria, tetanus, botulinus and staphylococcus toxins were all discovered towards the end of the nineteenth century, and so was the fact that they were antigenic and capable of being neutralized by their respective antisera. It is little wonder, therefore, that medical research at that time should have been domin-

(5) Polymer. *Many small molecules joined together to form a bigger molecule.*

(6) Neurotoxin. *A toxin that attacks the nervous system.*



(1) Dysentery toxin destroys marrow blood cells, resulting in the destruction of the nerve cells of the central nervous system: (2) Tetanus toxin acts on the inhibition synapse in the central nervous system; (3) botulin toxin acts on the neuromuscular junction of the peripheral nervous system; (4) central nervous system; (5) peripheral nervous system. (After Van Heyningen, Ann. Institut Pasteur, Paris).

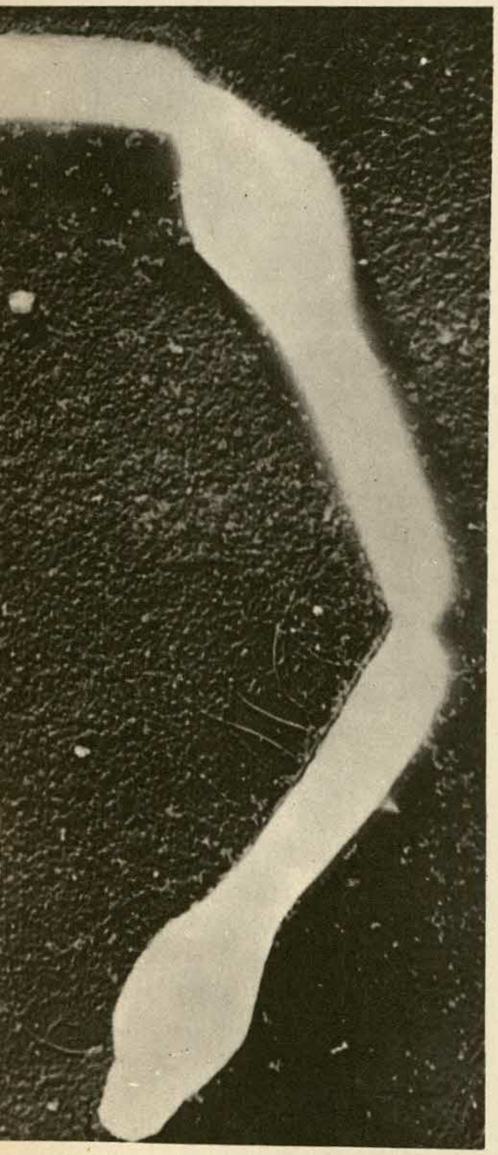
(7) Central nervous system. *Essentially the brain and spinal cord.*

(8) Peripheral nervous system. *Nerves carrying sensory and motor impulses between the central system and the limbs and organs.*

(9) Motor nerve. *A peripheral nerve carrying an inhibitory or excitatory impulse to a muscle.*

(10) Synapse. *The region of contact between two nerve cells, forming a place where the nervous impulse is carried from one nerve cell to another by means of a chemical transmitter.*

(11) Antiserum. *The clear fluid of the blood of an animal that has been immunized. The serum contains antibodies of the immunizing substance.*



Clostridium botulinum, *producer of botulin exotoxin, in advanced phase of division. The two cells contain a spore (unicellular means of dissemination) visibly distorting the bacterial body (mag. × 15,000. Istituto Superiore di Sanità, Rome).*

TABLE

The potency of bacterial exotoxins, compared with that of endotoxins and other poisonous substances.

TOXIC SUBSTANCE	Size of molecule	Presumed lethal dose for man (milligrams)	Toxicity compared with strychnine
Strychnine	small	40	1
Arsenic	small	1,400	0·03
Spheroidin (fish poison)	small	1	40
Crotactin (snake venom)	large	4	10
Ricin (plant poison)	large	2	20
Bacterial endotoxins (all species)	large, complex, but small toxic component	400	0·1
Bacterial exotoxins: α-toxin of Cl. perfringens	large	0·2	200
Diphtheria toxin	large	0·02	2,000
Dysentery toxin	large	0·000,06	700,000
Tetanus toxin	large	0·000,06	700,000
Botulinus toxin	large	0·000,06	700,000

ated by the idea that the harmful effects of infectious diseases were due to toxins, and that treatment could be simply effected with the appropriate antisera.[11] These hopes were soon dashed; we now know that only three or four diseases are entirely and unequivocally due to exotoxins, and that they can be exactly reproduced by the introduction of the germ-free toxins. These are botulism, tetanus, diphtheria and the red rash of scarlet fever. Botulism is a rare disease in humans, especially since the introduction of rigid sterilization control in the food preserving industries (in Great Britain there have been only eight deaths since medical records were made). It affects cattle to an economically important degree in South Africa, where it is now controlled by immunization. Tetanus and diphtheria have been largely eliminated from countries where active immunization is practised. (This modestly publicized fact is one of the greatest triumphs of preventive medicine). The red rash of scarlet fever is alarming for parents to see, but not dangerous for children to experience—in any case scarlet fever is now comparatively rare. It is quite possible, as a result of research during the war, that gas gangrene, a disease mainly affecting soldiers wounded in battle, can now be prevented by immunization against the toxins produced by the causative organisms (notably the α-toxin of *Clostridium perfringens*). Toxin-producing bacteria related to the gas gangrene group of organisms cause a number of diseases in sheep and calves, and these are now controlled by immunization. As to the many other known exotoxins, it is likely that they play a part in infectious disease, possibly an important part, but generally it is difficult, if not impossible, to define their role. For example, the staphylococci, streptococci and pneumococci produce many exotoxins, but immunization against these toxins appears to have little effect on the course of infection.

Toxins and biological warfare

There are even more sinister applications of exotoxins. A German bacteriologist recently murdered his wife by putting a trace of tetanus toxin in her tooth-paste and giving her a new hard-bristle tooth-brush. But for certain mistakes that he had made, he would not have been discovered, since the toxin, besides being lethal in undetectable amounts, is protein and therefore indistinguishable from the protein of the body. On a larger scale, the immense toxicity of botulinus toxin has tempted various powers to consider its possibilities as an agent in warfare. Botulinus toxin certainly appears at first sight to have several advantages as a genocidal agent. It could kill the enemy without destroying his property, and it is incomparably easier and cheaper to produce than a hydrogen bomb. With a little determination, a brewery could be converted within a few weeks into a factory capable of producing enough toxin theoretically sufficient to destroy many millions of people. The immense potency of this toxin should not blind us, however, to the realities of the situation. In warfare it would not be possible to inject the toxin into the enemy (except by using poisoned bullets, which could not be used on a massive scale). The toxin would therefore have to be dispersed in the air, or sprayed on crops, or placed in water supplies, and thus enter the enemy through the nose, lungs, skin or mouth. We have seen that by these routes its potency is no greater than that of the small-molecular poisons and thus the one strategic property, its extreme theoretical potency, would be unrealizable in practice. And if its potency is not an advantage, its other properties are a disadvantage. Since it is labile it would rapidly deteriorate if dispersed in the air, and it could be easily destroyed in water supplies by boiling the water with a pinch of soda. And as it is antigenic it should be possible to immunize the population against it. This would need some effort and organization, but not nearly as much as the provision of modest air-raid shelters. As an agent for genocide botulinus toxin is therefore impracticable, and despite some newspaper reports, it is not a serious threat to mankind.

Bacterial virulence

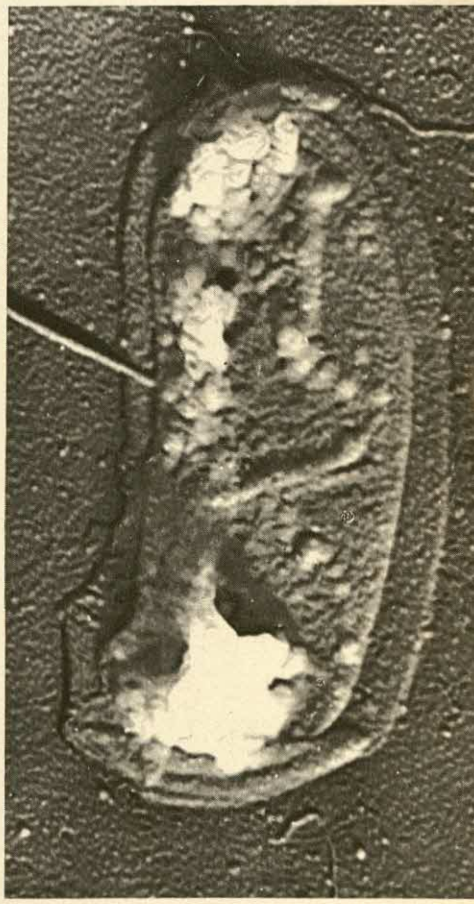
Albert DELAUNAY

Some bacteria can be tolerated by an organism without their causing it any damage: these are called saprophytes. Other bacteria are capable of causing lesions of greater or lesser extent in the same organism: these bacteria are said to be virulent. The virulence of a germ seems to depend on certain characters of its structure, and on the biochemical characteristics of the infected tissues.

Germs are everywhere. Innumerable kinds live in the air we breathe, in the earth we tread, and in the waters, fresh or salt, which surround us. The infant is born uncontaminated by germs, but within a few hours of birth, micro-organisms begin to spread and multiply within his body and for the rest of his life will live with him as commensals.

Bacteria are by no means always the enemy of man; on the contrary, as we have already seen, he must often be thankful for their ubiquity. Breaking down all dead organic material, they carry out a considerable work of 'cleaning-up' on the earth's surface, and this, added to the work of plants and animals, ensures the completion of the great cycles run by essential chemical elements: carbon, nitrogen, sulphur, phosphorus, etc, enabling life to continue.[1] Also we must not forget that certain micro-organisms are valuable partners in the work of the farmer and the industrialist. Furthermore it is possible that some of the germs we normally carry within ourselves might perhaps be responsible for important metabolic reactions.

Having pointed out the good which is done by many micro-organisms, we must not lose sight of the fact that some of them, be they bacteria or viruses, are formidable adversaries for both man and animals. These are the pathogenic, in contrast to the generally beneficial saprophytic micro-organisms, and in fact they are capable of causing severe, if not deadly, diseases.



A cholera vibrio in an advanced state of autolysis. (Mag. × 42,000. Istituto Superiore di Sanità, Rome).

What is virulence?

What makes a micro-organism pathogenic? This is a big question and one which has been asked since the time of Pasteur. In general, the following reply may be given: microbes may be pathogenic (*a*) when they have the power of producing the particular poisons known today under the name of exotoxins; (*b*) when they are virulent.

The first part of the reply is satisfactory and many remarkable examples can be quoted here, such as the bacilli of diphtheria, tetanus and botulinus. In each case the germs remain at the place where they enter the organism and multiply only feebly. The danger comes from the exotoxin which they set free and which diffuses throughout the body, seriously affecting the working of cells or chemical constituents which are particularly vulnerable. When the lesions are widespread, it is not surprising if they lead to death.[2]

On the other hand, when we consider the second part of the reply, concerning virulence, the position is not nearly so clear. If one looks at definitions of virulence in the standard textbooks of bacteriology one finds unsatisfactory statements such as the following: 'Virulence is the ability of germs to multiply in infected tissues.' Though true enough, this is also very vague. Let us try and throw a little light on the subject. For a germ to be virulent three conditions, at least, must be fulfilled:

1. The germ must possess particular constituents;
2. it must obtain access to tissues which are able to provide it with the factors necessary for its life and reproduction;
3. the host tissues must suffer from the presence of the pathogen, whether the lesion produced is localized (an abscess) or general (septicaemia).

The biologist who is occupied with the problem of virulence must therefore consider the part played by the microbe and that played by the organism which is infected. Virulence is not a property of the germs only, as is the production of an exotoxin, but rather the result of an interaction between the two organisms, on the one hand, the microbe and on the other, the host suffering from the infection.

How can virulence be explained?

To start with we will consider virulence from the bacterial aspect. We have already said that to be virulent a germ must of necessity possess certain characteristics in its make-up. Some light has been thrown on this aspect by the work of Boivin and his collaborators (1935–49), who showed that among the Gram-negative bacteria (a group which includes among others the typhoid bacillus and the colon bacilli), the only ones which are virulent are those which contain a special substance called lipopolysaccharide.[3]

How does this substance act as so redoubtable a weapon for these bacteria? It now appears that its mode of action is a double one. On the one hand, it confers on the germs which carry it a raised resistance to phagocytosis by the protective cells of the body; on the other, once it has been liberated into the infected tissue from germs which are breaking up, it is capable of disturbing, to a greater or lesser extent, numerous metabolic activities in the host's cells.

However, we do not yet have the whole solution to the problem. For example, if we inject a typhoid bacillus, one which carries lipopolysaccharide, into a mouse or a guinea-pig, these animals will at most suffer some poisoning due to the liberation of a certain quantity of lipopolysaccharide into their bodies, but they will not get typhoid fever. In spite of the presence of the lipopolysaccharide, the bacteria are not virulent, and they show no capacity for multiplying in the tissues. Thus the presence of this substance in the bacteria is only a partial explanation of what happens. As Boivin himself said, 'Its presence is a necessary condition for bacterial virulence, but it is not of itself a sufficient condition.'

In any case, this is true only of Gram-negative bacteria. No similar substances are known in the Gram-positive bacteria such as streptococci. Nevertheless, it is well known that these germs can be equally dangerous, but we do not yet know what are the particular properties that confer virulence in Gram-positive bacteria. It seems probable that the pathogenicity or, more exactly, the virulence of these organisms must also be constitutional, that is, they depend on a certain 'arrangement' of their molecules. It does happen that certain Gram-positive germs, if they are kept for some time *in vitro* lose their virulence for a particular animal; during the course of repeated sub-culture they have become modified. The bacteriologist has moreover found ways of starting, or at least of promoting, such changes. These include the action of light, of temperature, of desiccation, etc, and we need hardly recall to mind in this connection the famous experiments of Pasteur.

(1) *See the chapter, 'The role of saprophytic bacteria' by Carlo Arnaudi, page 68.*

(2) *See the chapter, 'Bacterial exotoxins' by William E. van Heyningen, page 79.*

(3) *See the chapter, 'The importance of endotoxins' by Albert Delaunay, page 27.*

(4) *See the chapters on 'The L forms' by Paul Hauduroy and 'The protoplast' by Giulio A. Maccacaro, pages 41 and 46.*

But it is not enough to say that there has been a change in the bacterial cell. One would like to be able to say what has changed, or even been lost. Is it a major component of the cell, or an enzyme, or something else? Unfortunately, as yet, there is no definite information; but a certain enzymatic equipment is undoubtedly of importance in the changes. It has been shown, for example, that the large majority of virulent staphylococci possess a coagulase. This coagulase makes the germs which possess it more resistant to phagocytosis. It is evident that such germs will be more virulent.

Other constituents of the bacteria which have no enzymatic action may also play a part, in particular those which make up the bacterial walls, or in some cases their capsules. This has been well shown during the past few years by the work on L forms, and on protoplasts.[4] We think now that the constituent which confers virulence on bacteria must be found often, if not always, on the *surface*. The surface is important because it denotes the area of contact, or the place where the germ and its environment meet. In the case of a bacterium causing an infection it is the host organism which is the environment.

The part played by the host

The role which is played by the host organism is very considerable, and can be demonstrated in many ways.

(*a*) Any germ is very unequally pathogenic for different species of animals which it can infect, either naturally or in an experiment. For instance, the rabbit is less susceptible to the anthrax bacillus than is the guinea-pig. The glanders bacillus can multiply in the horse, in the ass and in man, but gives rise to different disease symptoms which may be either acute or chronic.

(*b*) Any one animal species is susceptible to certain species of germs, and refractory to infection by others. Here again there are plenty of examples to choose from. The dog, which picks up rabies very easily, is quite resistant to anthrax. The rat, which is equally unaffected by anthrax, is nearly always defenceless against the plague bacillus.

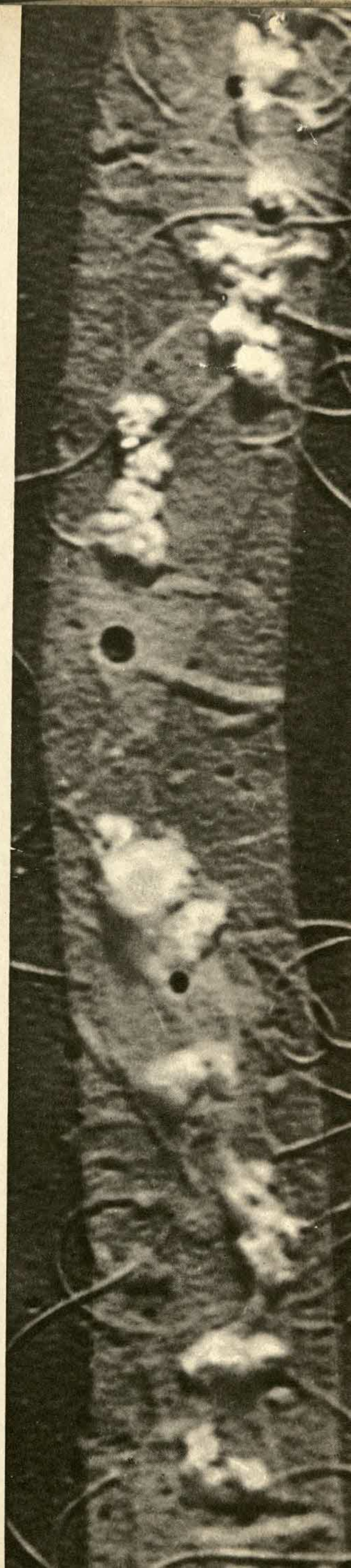
(*c*) Closely related species often have similar susceptibilities to infection. Thus most of the animal infections which are transmitted to human beings come from other mammals. The great apes which are our close relatives are usually very susceptible to human diseases; it has been possible to give chimpanzees syphilis, poliomyelitis and exanthematic typhus, all notorious scourges of the human race. However, this rule must not be pressed too far; it is in fact impossible to be quite certain in advance whether a given bacterium or virus will be pathogenic for a particular species. One can reply to this question only after experiment.

(*d*) We must also take account of race. The facts show, for example, that the Negro races are very susceptible to tuberculosis but that they resist yellow fever or malaria better than do whites. Exanthematic typhus is more severe in Europeans than among Arabs, while scarlet fever may be exceptionally severe in Anglo-Saxons.

(*e*) Even between individuals difference in susceptibility occurs. Guinea-pigs inoculated with the same dose of tubercle bacilli do not all die within the same time, especially if the dose is low. Among human beings the virus of poliomyelitis produces its ravages only in a minority of people.

(*f*) Besides differences between groups of animals, between species and between individuals, age is also an important consideration. Usually it is young people who are more susceptible to the pathogenic agents; the gravity of tuberculosis in young infants is well known. The mortality from whooping cough is especially high in infants of from six months to one year. After puberty the hair is no longer susceptible to ringworm.

Why these differences? Clearly it is because an illness is produced only when a certain relationship has been established between the attacking organism and the host. Nicolle once compared this relationship to that between a lock and the corresponding key. It seems fairly certain that this relationship is of a chemical nature. To cause disease, a bacterium must not only be able to survive and to multiply in the infected organism, but must also succeed in damaging its host by means of the constituents of its own surface layers, or by the chemical products which it liberates either during its life or when it breaks up after death.



Clostridium tetani *secretes a very virulent toxin. Inside the cell wall there are numerous corpuscles (the white masses) from which the bundles of cilia take their origin. (Mag. × 21,000. Istituto Superiore di Sanità, Rome).*

The resistance of organisms to bacteria

We have talked of a relationship but it would be more exact to talk of an equilibrium. All gradations exist between the immediate and vigorous rejection of the aggressor micro-organism, and a fatal disease. Often, in fact, for several days the outcome may be uncertain. The two organisms placed face to face seem to be watching each other. Something else than mere juxtaposition seems to be needed for the illness to develop; in other words, for the organism that has been attacked to succumb. This is well established from clinical facts but is perhaps even better shown in data from the laboratory. Many experimental means are already available, even if we leave out the specific methods such as vaccinations or serum treatments, which will either augment or diminish the normal resistance of an animal to a pathogenic organism (or, we could equally say, the virulence of a pathogenic germ). Among these ways we may list the following:

The giving of special diets (rich or poor in vitamins, for instance); the injection of hormones (cortisone, for example); the production of traumatic shock or the giving of ionizing radiations (such as x-rays); administering various substances at the same time as the infecting organism, such as mucin or polypeptides.

One case of such administration is of particular interest; it is that of a substance we have already had occasion to mention, the lipopolysaccharide derived from Gram-negative bacteria. If this bacterial constituent is purified, redissolved in water and injected into animals such as mice or guinea-pigs in non-lethal doses, it is found that the resistance of these animals to microbes in general is markedly affected. At first the susceptibility is increased. Later, on about the third day, this effect is reversed and the resistance is increased. How does the injection of a small dose of bacterial lipopolysaccharide first diminish and then increase the non-specific resistance of an animal to infection?

At first antibodies were supposed to be involved, then phagocytes. Then in 1955 Pillemer, an American biologist, announced the discovery of 'properdin', and this was thought to be the answer. Properdin is a special bactericidal substance which has been isolated from blood serum. The level of properdin in the blood is variable and is depressed immediately after an injection of lipopolysaccharide; after three days it rises to above the normal level. The coincidence was certainly striking, but further experimentation showed that the level of blood properdin was not always related to susceptibility.

At present we may say that the hypothesis which is best supported is the one which suggests a link between the non-specific resistance to infection and certain metabolic processes; or to put the situation more clearly: the modifications in resistance with which we are dealing are in the end probably due to a cellular activity which, on the metabolic plane, has become abnormal. Where metabolic activity is normal, a given bacterium may or may not be pathogenic, that is to say, it may or may not be able to display virulence. But if metabolic activity takes a different course, then the behaviour of the bacterium also may be totally changed, in one direction or in the other. The evidence for this has been building up for some time.

First of all it was ascertained that the bacterial lipopolysaccharide which effects such a marked change in host resistance is also one of a number of substances which are capable of changing many cellular metabolic activities. For example in our own laboratory we have shown that in animals treated with these substances sugar metabolism is profoundly altered. We have reason to think that these changes in metabolism and in resistance to infection may be closely linked.

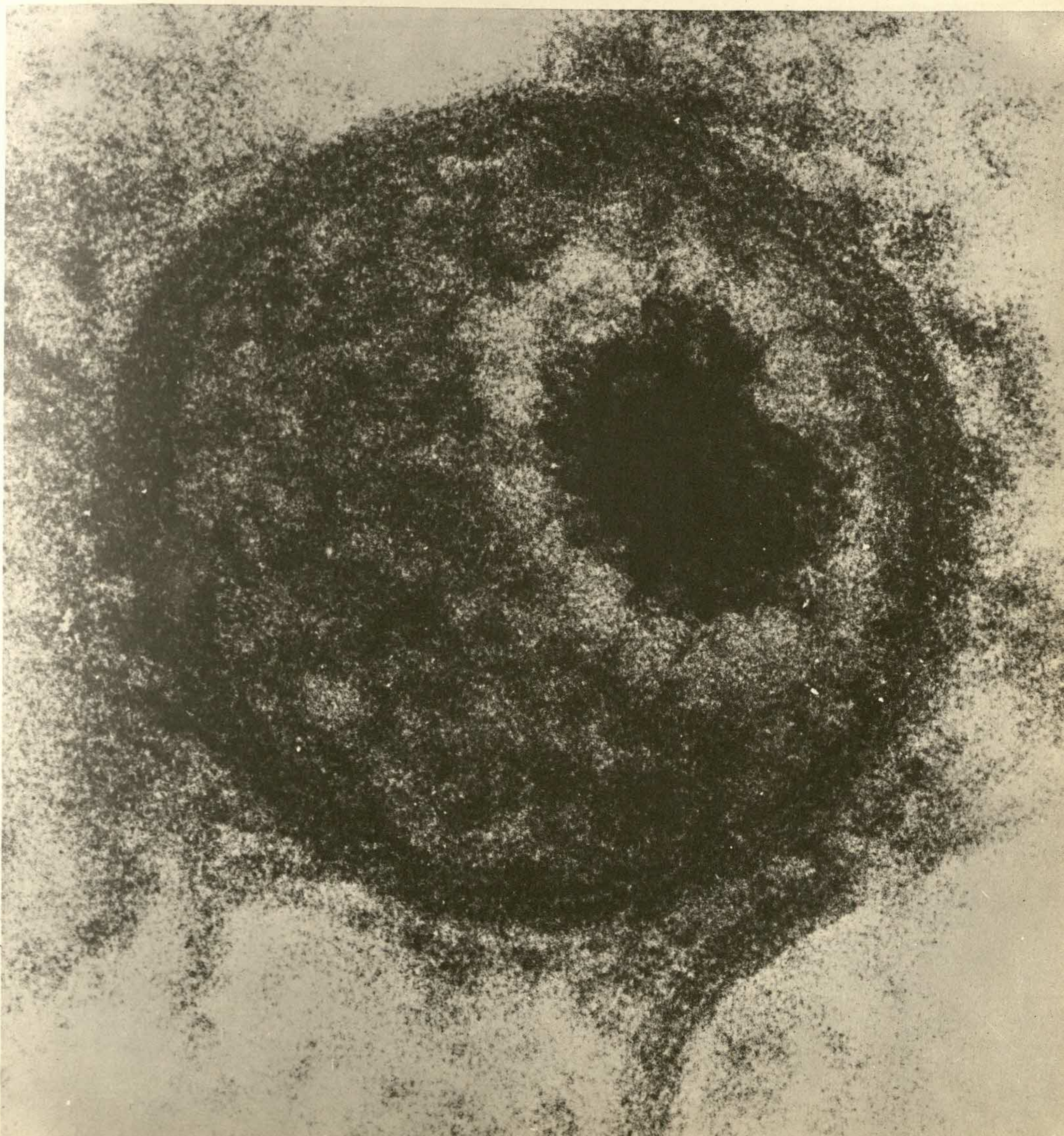
An example of an abnormal metabolite affecting resistance is 'interferon'.[5] This work is of the greatest interest. The accumulation of certain normal metabolites can cause changes in the resistance. Urea and lactic acid are examples of normal metabolite involvement, but also of considerable interest are the ketones. It is known that the spores of fungi responsible for the disease mucormycosis will not germinate in normal rabbits. But if rabbits are rendered diabetic by the injection of the chemical alloxan then the spores will germinate and cause the disease. This germination does not depend solely on a raised level of glucose in the blood. It occurs only during the short period in which the animal suffers from acetonaemia, that is, the presence of the ketone, acetone, in the blood.

Everywhere these days we come back to biochemistry for the explanation of vital phenomena and this is true of bacterial virulence. Biochemistry is certainly best placed to reveal to us the truth about what has been known, since the time of Pasteur, as the 'soil' in which disease is sown.

(5) *See the chapter, 'Interferon' by Alick Isaacs, page 139.*

PART FOUR

Viruses



Virus of Shope fibroma, magnified 500,000 times. In the centre is the nucleotide containing the genetic material of the virus (Photo Dr W. Bernhard, Institute of Cancer Research, Villejuif, France).

What is a virus?

Luigi CALIFANO

Is a virus a living being, fully capable of reproducing itself, or an inanimate substance that can be reproduced only by the cell it has infected? This question has been debated since the discovery of the first virus. Reasons are given in this chapter which argue in favour of the first hypothesis. Nevertheless, the virus would still be a living being, unique in its origin, characteristics and behaviour.

Pasteur first used the term virus to describe agents responsible for infectious diseases, such as the anthrax virus, tuberculosis virus, etc. Gradually, as the germs were identified, they were given specific names: *Bacillus anthracis*, *Mycobacterium tuberculosis*, etc. However, the aetiology of a certain number of infectious diseases remained unknown and for these the general term of virus continued to be used.

In 1892 Ivanovsky made a fundamental discovery: a disease which attacks the tobacco plant and which is called 'tobacco mosaic' could be transmitted by a filtrate of the juice of the tobacco plant leaves after passage through a porcelain filter. Independently of this observation, Beijerinck, in 1898, obtained similar results and gave full significance to the agent by qualifying it as *contagium virum fluidum*. From this was born the term 'filterable viruses', which serves to describe the infectious agents which are invisible even under microscopes providing the highest magnification. Viruses, unlike bacteria, cannot be grown on inert culture media; they are smaller than the smallest known bacteria.

Loeffler and Frosch established in 1898 that the liquid from the vesicles of distemper contained an agent which had properties analogous to those of the tobacco mosaic virus. Other discoveries followed concerning virus infectious diseases of man, animals and plants. The term filterable virus passed into general usage, despite its inappropriateness.

Cellular inclusions

An early important discovery was the demonstration of intra-cellular 'inclusions' found in certain virus diseases: inclusions discovered by Negri in the nerve cells attacked by the virus of rabies, and inclusions or Guarnieri bodies produced by the virus from the vaccination.

Inclusions are sometimes cytoplasmic[1] and sometimes nuclear, depending on the virus and host. In certain diseases it has been possible to mark, thanks to special colouring methods and very high optical magnification, minute granules sometimes associated with inclusions. These are called 'elementary cor-

(1) Cytoplasm: *living matter contained within the cell (excluding the nucleus).*

(2) Millicron (mμ): *a millionth of a millimetre.*

(3) Enzymes: *specific organic catalysts for different chemical reactions.*

(4) *In connection with this subject see the chapter on 'The bacteriophage', page 106.*

puscles' and are thought by some to be virus particles.

In 1931 Woodruff and Goodpasture demonstrated that it was possible to cultivate a virus inside a living fertilized chicken egg. This technique helped very much in the rapid discovery of other viruses and made it possible to obtain large masses of an infective material.

Perhaps the most important discovery made in virology, and one of the most famous in biology, was Stanley's success in obtaining the tobacco mosaic virus in the form of crystallizable material. Eventually it became possible to crystallize other viruses responsible for plant and animal virus diseases—among the latter being the equine encephalo-myelitis virus. In 1955, Schwerdt and Schaffer succeeded in crystallizing the poliomyelitis virus, which is among the smallest. In 1956, due to the work of Mattern and Du Buy, the coxsackie virus was crystallized. Naturally, the possibility of working on crystallized viruses has led to research which would not otherwise have been effected.

Another significant step was the use of the electron microscope. Here again the tobacco mosaic virus gave rise to the discoveries which are owed to three German research workers—Kausche, Pfankuch and Ruska—who used the first electron microscope as constructed by Ruska's brother, a physicist. The virus of tobacco mosaic was revealed as a short rod.

Present-day techniques, using highly improved electron microscopes, not only supply the shadowed image of the virus particle but allow calculation of its actual size and comparison between different virus particles.

The most decisive technical advance was when Enders discovered the possibility of cultivating viruses in cells maintained *in vitro*. These discoveries enabled fundamental and positive characteristics of the virus to be determined which before could be defined only in negative terms: their invisibility under the optical microscope; their failure to grow on inert media; the absence of morphological structure; and power of penetrating bacteriological filters.

Today, we know that the virus has the following characteristics: (1) a definite morphological structure visible under the electron microscope; (2) a chemical nature all its own; (3) a need to enter into living cells, that is to say, metabolizing cells, in order to reproduce; (4) a size of the millimicron[2] order, and which varies according to the virus, the smallest having a diameter of little more than 10 mμ and the largest reaching up to 500 mμ.

The shape revealed by electron micrographs is often very simple: a rod for the tobacco mosaic virus, spherical for the poliomyelitis virus, and hexahedral for that of the vaccine.

The bacteriophage, which is the virus of bacteria has, on the other hand, a more complex morphology.

Chemical constitution of viruses and their action on cells

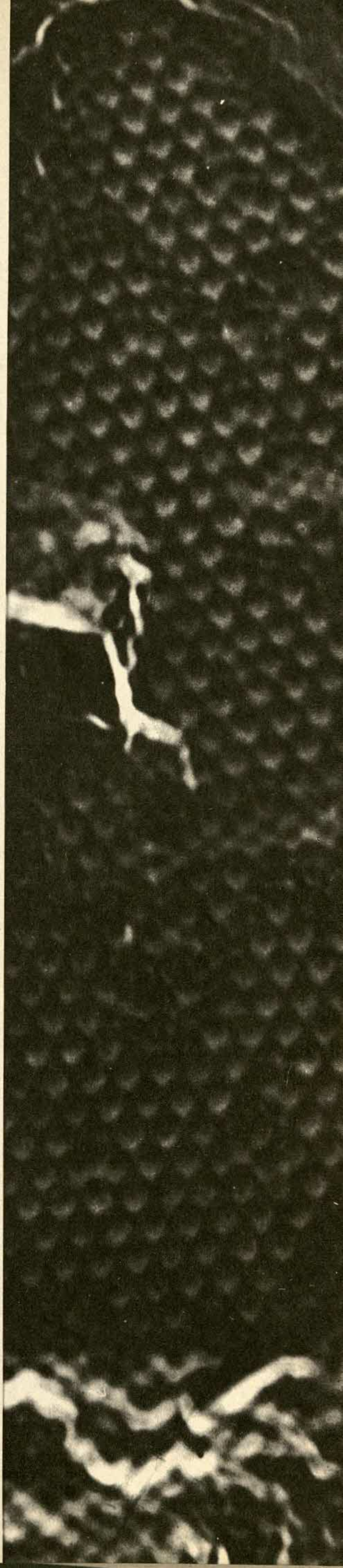
The chemical constitution of viruses has been the object of much research and if everything has not yet been clarified, the essentials are already known. All viruses are principally composed of nucleoproteins, that is to say, a combination, in clearly defined proportions, of protein and nucleic acid. The protein is of the basic type. The nucleic acid component may be one of the two types found in all living beings, with small variants. Most often it is deoxyribonucleic acid, but certain viruses of animal diseases contain ribonucleic acid instead of DNA.

In some viruses other constituents, such as lipids, traces of copper, etc, may also be present.

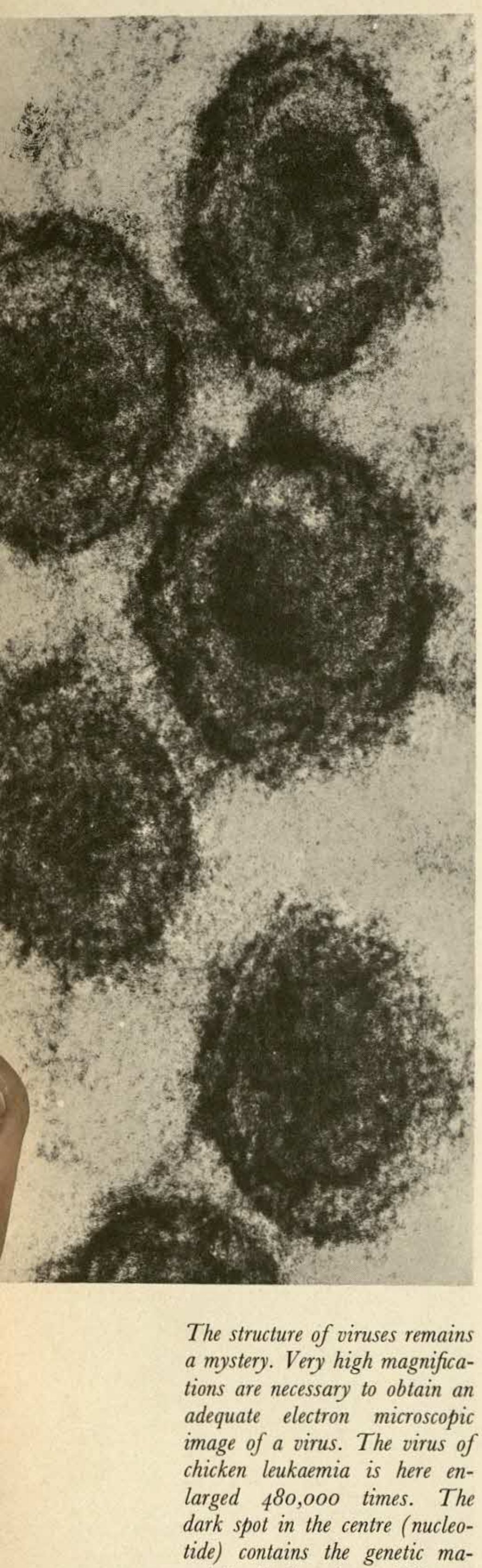
A particular characteristic, one of considerable significance for biology in general, is that viruses contain no enzymes;[3] they cannot provoke any metabolic reaction. During the last few years, however, a small enzymatic activity has been reported for a few viruses. It is probable that the distal end of the tail of the phage is equipped with enzymes capable of reacting with certain constituents of the bacterial wall. Certain viruses can agglutinate red cells, a fact attributed by many to the existence of an enzyme acting on the constituents of the enythrocyte membrane. These are, however, isolated and limited phenomena.

It remains certain that viruses are utterly devoid of enzymes, a situation that can be summed up by the remark, 'Viruses do not have a proper metabolism'. This statement poses the problem of the biological nature of filterable viruses, whether they can be considered as living beings. We will return to this subject.

The third characteristic of viruses is their need to enter a living cell in order to multiply. There is no doubt about this requirement.



The electron microscopic image of the virus which causes turnip jaundice. (*Mag.* × *85,200, I.N.R.A.*).



The structure of viruses remains a mystery. Very high magnifications are necessary to obtain an adequate electron microscopic image of a virus. The virus of chicken leukaemia is here enlarged 480,000 times. The dark spot in the centre (nucleotide) contains the genetic material of the virus. (Electron micrographs by Dr W. Bernhard, of the Institute of Cancer Research, Villejuif, France).

The manner in which the cells are broken into is well known. It has been particularly well studied in the case of phages.[4]

Viruses, developing intracellularly, can bring about the death of the host cell, as in the case of the pathogenic viruses. They can also fail to produce any appreciable modification of the host cell—such are the 'latent' viruses. Yet again, viruses can provoke the proliferation of cells, as with the virus of mammary tumours and the virus of leukaemia.

The morbid damage relating to each virus disease can be traced back to the cytopathogenic[5] effects. Paralysis in poliomyelitis is the consequence of the cytopathogenic effect of the virus on the nerve cells; because these are unable to regenerate, the effect is permanent. The mottling of the leaves of the tobacco plant reflects the cytopathogenic action of the virus. One can interpret in the same way the lysis of the bacterial cell by the phage. A cytopathogenic effect even occurs in infected cultures *in vitro*.

Dulbecco noticed that viruses grown in cells cultivated *in vitro* on agar caused zones of necrosis which were easily visible to the naked eye and could be used to make a quantitative determination of the amount of virus present.

Latent viruses ('orphans'), first discovered in plants, such as the latent virus disease of potatoes, have a great importance in pathology. They are also found in animals and man. It is possible to isolate them and to cultivate them *in vitro*. A great many viruses latent in one species may be cytopathogenic in hosts of different species. This has been demonstrated *in vitro* and *in vivo*.

The latent virus of the disease of potatoes was, in fact, discovered by the effect it has on tomatoes, in which it produces a disease resembling that of the tobacco mosaic; in potatoes—where it reproduces abundantly—it is not pathogenic.

Reconstruction and crystallization

It is now time to say a little about a fact which, at one time, led to some very lively controversy: the reconstruction of viruses. It had been known for some time that it is possible to fragment viruses into smaller particles and—under certain favourable conditions—to effect some kind of recombination of these particles. However, such a recombined virus always showed itself inactive from the biological point of view.

Conrad was the first to succeed in dissociating and reconstituting a virus in its active form, an achievement which has been interpreted by some as a first step towards the synthesis *in vitro* of virus particles.

Great progress has been made in the purification and crystallization of viruses. If the crystals of the tobacco mosaic virus are not true crystals but only para-crystals—such as was shown by Bawden and Pirie, as a result of their röntgenographic research—a true crystal has been obtained with the virus of ***bushy stunt***, the mosaic virus of tomatoes, which behaves absolutely homogeneously in physical tests.

In this respect one should note that the term 'crystallized virus' does not yet mean true molecular crystallization, but the aggregation of virus particles in such a way as to form a crystalline structure.

This means that a virus is not a simple molecule but that it has a structure presenting a base of morphogenesis that can be developed to a greater or lesser extent, as the case may be. One thus acknowledges in viruses a certain property of organization. Instead of calling them protein viruses—as was originally done by Stanley—it would be better to call them nucleoprotein viruses, the essential part active in infection and multiplication being nucleic acid.

Are viruses living?

Since Stanley succeeded in crystallizing the mosaic virus, biologists have asked themselves whether the virus was living or non-living, from whence it came, and finally, what was its relationship to the inorganic world on one hand and the world of living beings on the other.

The first question gives rise to another: 'What is life?' It is necessary in this respect to distinguish between living substance and a living organism. All organisms are formed from living substance, but this in itself does not constitute an organism. The cytoplasm of a bacterial cell is living substance, but it requires cellular organization before one has a unicellular organism. In the same way, the organization of cells in tissues, and of tissues in organs, according to the development plan peculiar to the species, is necessary for a multicellular being (metazoan or metaphytic). The concept of living substance is more difficult to define. It is certain that only a living thing can transform energy obtained either from outside itself or from chemical sources, organic or inorganic. If one accepts this idea, the chloroplast[6] which fixes the energy of light is living, just as one can say that other cellular structures such as mitochondria, which liberate chemical energy from a substrate, are living. The energy thus available is used for different cellular functions, first of all for the synthesis of new molecules identical with pre-existing ones. This indicates that molecular division is an attribute of life and that for this to be accomplished complex metabolic phenomena are indispensable.

If molecular division is characteristic of living substance, it follows that reproduction, which is possible only on the basis of molecular division, is a characteristic of living organisms.

If the organism is unicellular its reproduction corresponds to the reproduction of a cell; if it is multicellular, cellular multiplication takes place in several of its tissues, but the reproduction of the whole organism is accomplished only at the level of specially differentiated cells.

Metabolic phenomena are dependent upon enzymes, the formation of which is not autonomous but is determined or directed by genes.[7] Consequently, an organism is above all a carrier or transmitter of inheritance; everything that takes place in the life of a cell is part of this property.

Do the genes represent a living substance? If one takes the view that life cannot be separated from metabolism, then genes are not living substances. However, since the existence of living substances seems to be impossible without the presence of genes, one is obliged to conclude that life is in the first place a genetic substance, hence metabolism, then structure and from there, organism.

What is the position of filterable viruses in relation to what precedes? A virus has no proper metabolism, but is an exclusively intracellular parasite and, of the different activities of the organism, such as metabolism, reproduction and genetic activity, it possesses only the last.

The nucleic acid of the virus, when introduced into the cell, functions as a particle of genetic equipment which mingles with that of the cell. The cell is withdrawn, so to speak, from the influence of its own genes and submits to the action of those of the virus. In this new situation the genes which are reduplicated by the cellular metabolism are those of the virus and not those of the host cell; consequently, the number of virus particles multiplies, and the cell dies because it cannot form the enzymes that are indispensable for its metabolism. The virus genes behave in a dominant fashion. It is only in the case of the latent viruses that 'cohabitation' becomes possible in the sense that the host cell reduplicates both the virus and its own genes, and is therefore capable of normal metabolic activity.

Only the nucleic acid of phage penetrates into the bacterial cell, but mature phage particles are later liberated; these possess a protein envelope, a head, a tail and a fringe, that is to say, a proteinic organization around the nucleic structure, with the beginnings of differentiation.

Since the virus shows the outlines of a differentiated structure, it can perhaps after all be considered an organism. Certainly, the components of this structure are not formed by themselves but by the host cells; but they are nonetheless the beginnings of differentiation and are the expression of the transmission of hereditary characteristics.

In conclusion, viruses can be defined as elements possessing a molecular structure of biological origin and which are capable of intervening in the nucleoprotein synthesis of the host cell, inducing it to differentiate the structure of the virus.

The question of the origin of viruses can be approached only by induction. Certain research workers, like Green and Laidlaw, consider the virus as a parasite which has evolved retrogressively, the virus of today deriving from micro-organisms which became more and more adapted to intracellular parasitic life until they lost all, or nearly all, their own metabolic activity. They can be thought of as mutants of micro-organisms that have gradually lost all, or nearly all, the genes which determine metabolism, conserving of their ancient genetical inheritance only that part which serves to determine their division.

Those who hold the opposite view emphasize—not without reason—that viral parasitism is highly specific, not only at the level of the species but also at that of the tissues; that is to say, many viruses are parasites of only one type of cell in one type of species. They conclude from this that the appearance of a virus can only be autochthonous, that is, derived from the genetical inheritance of the host cells. Future research will undoubtedly confirm one or other of these concepts or reject both.

At the present time, what is certain is that the virus does not represent one of the primordial forms of life. Its reproduction, as we have seen, depends on that of its host cell, so that its appearance could not have occurred until after the appearance of its susceptible host.

(5) Cytopathogenic: *a virus that produces lesions in the cytoplasm and in the cell.*

(6) Chloroplasts: *small green corpuscles peculiar to cells of green plants; they are carriers of chlorophyll.*

(7) Genes: *the factors that transmit hereditary characteristics. They are composed of molecules of deoxyribonucleic acid.*

Viruses in the laboratory

Claude HANNOUN

Less than thirty years ago the place occupied by virology was very modest compared with that held by bacteriology. Today, the opposite applies. Why was virology so far behind? It was because particles, which are of very small size, were for a long time particularly difficult to study. However, in this field, as in many others, human ingenuity overcame the obstacles and opened the door to knowledge. Now we are able to maintain living viruses, identifying them, and studying their properties: this chapter describes the fundamental discoveries made in virology and their first application.

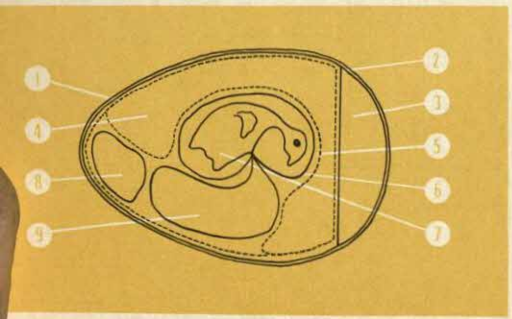


Fig. 1. Schematic section of a chick embryo incubated for ten days. (1) shell membrane; (2) chorio-allantoic membrane; (3) air-space; (4) allantoic fluid; (5) amniotic membrane; (6) amniotic fluid; (7) embryo; (8) yolk sac; (9) albumen.

The term virus, which, before the discovery of microbes, described all agents capable of transmitting disease, was later used in the sense of 'pathogenic germ'. Today it stands as an abbreviation for 'ultrafilterable virus' or 'ultra-virus', and has thus taken on a more limited meaning. Viruses are minute, pathogenic agents capable of passing through the very fine filters which hold back bacteria. They were invisible until the limits of visibility imposed by the optical microscope were overcome.

Viruses hold a place apart in the world of microbes. Even though seventy years ago their existence was hardly suspected, we know nowadays that they form an important group of dangerous pathogenic agents against which man still only knows how to defend himself very imperfectly. The biological properties of viruses are of very special interest because they pose problems which touch on the most mysterious functions of cells.

Viruses do not multiply by assimilation, growth, and then division, like bacteria: they reproduce only as parasites on living cells. Consequently all the operations of isolation, identification and culture of a virus in the laboratory are possible only with living cells to serve as a host. Thus, the first problem in the culture and manipulation of viruses is to find a sensitive host-cell.[1] This is sometimes provided by a plant or an animal, by organs in survival, or by cells in culture.

Before going into details of the methods used, it is necessary to distinguish between two principal circumstances where identification is required: on the one hand, when the virus is known, and on the other, when it is unknown. The first involves diagnosis of a clinical effect which resembles that of a known virus disease,[2] and the isolation and identification of the virus in question. This is a diagnostic operation which is often relatively simple or commonplace, although it may involve delicate

(1) Sensitive cells: *living cells in which a particular virus can reproduce itself. Not all the cells of an animal are sensitive to a virus: each virus has a particular affinity for the cells of certain organs in certain animals.*

(2) Principal human diseases caused by viruses: *the most serious viral diseases in man are smallpox, rabies, poliomyelitis, yellow fever and influenza; commoner and less serious complaints include mumps, measles, chicken-pox, the common cold and viruses discovered more recently, the pathogenic roles of which are not always well defined, such as Coxsackie virus, Echo, adenoviruses and other myxoviruses (the group to which the influenza virus belongs). Viruses also attack plants, insects and bacteria.*

(3) *A characteristic example is that of the influenza virus, discovered during the epidemic of 1918. At that time, the demonstration of the existence of an influenza virus was made by inoculations in man, then in monkeys, whilst other laboratory*

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techniques. The second circumstance arises when it is necessary to demonstrate that a certain illness, the cause of which was previously unknown, is infectious and where the presumed agent answers more to the criteria which define a 'virus' than those which define a bacterium or a fungus. This will result in an important discovery and the proof may require innumerable experiments, with a great number of controlling factors. The two cases are very different, although common techniques are used.

Inoculation

To prove the infectious nature of a disease it is necessary to reproduce it in another member of the same species by placing the healthy one in contact with one bearing the disease, or by inoculating the healthy one with some material from the sick one (blood, saliva, a piece of a lesion, etc).

However, to confirm the presence of a virus it is necessary to verify that the material capable of transmitting the disease does not contain another pathogenic agent, in particular a bacterium; in other words, the material must be bacteriologically sterile. Then again, it must be capable of undergoing processes which viruses resist, for example, filtration through special membranes of collodion or asbestos, with pores so small that they are capable of passing particles of only a limited size (0·3μ, for example).

The identification of a virus by passage over a host of the same species is obviously difficult to carry out where human beings are concerned; nevertheless, those making the experiments have found volunteers willing to submit to the tests and to accept calculated risks. With benign infections there is not much danger, but it is never possible to know for certain what all the consequences of such an inoculation will be and this method therefore remains limited in its application.

The choice of an animal

An animal of a closely related species can also be used for test inoculation. Monkeys are sensitive to many human diseases, which they often contract naturally; thus it is possible to reproduce in monkeys the virus diseases of man, with the same clinical symptoms and comparable development. Certain symptoms may be difficult to assess, for example, cutaneous eruptions which cannot be easily seen, but the experimenter can estimate the activity of the monkeys, take their temperature, take blood samples, and submit them to various clinical examinations. The use of monkeys, however, presents some inconvenience: it is difficult to procure them in large numbers, and they are delicate and difficult to handle and do not do well in captivity. Other animals such as the rabbit, guinea-pig, rat or mouse, being more convenient, are sometimes used instead. It is necessary, of course, for them to be sensitive to the virus that is being studied, so in attempts to isolate a virus it is necessary to test its virulence on other animals. If one of these responds to the inoculation, then future studies are made easier.[3]

The chick embryo has been found to be convenient for the culture of many viruses. An egg embryo incubated for ten days is inoculated through the shell by means of a hole made by a sterile needle and is left to continue incubating. This technique, introduced into microbiology by Goodpasture, was much used and developed for viruses by Burnet. The virus can be placed on one of the three enveloping membranes or introduced into the liquid (amniotic fluid) which bathes the foetus (fig. 1).

The chick embryo is sheltered from the natural diseases often observed in laboratory animals; it can easily be manipulated under sterile conditions, and is sensitive to viruses which are harmless to the adult bird, such as the influenza virus. Also, the chick embryo possesses few natural factors of defence, specific or non-specific, that sometimes intervene and prevent passage in adult animals. It is important to note that viruses are often much more virulent against young organisms than against adults.

The chick embryo reacts in general to viral infection by lesions which are non-specific and difficult to see, except when the reaction occurs on the chorio-allantoic membrane, when the lesions appear as small whitish pustules. After a short period of development, an embryo inoculated by other routes dies and this can readily be seen by holding the egg up to the light. The presence of virus in the embryo or extra-embryonic liquids also shows itself in other ways; for example, by the appearance of a haemagglutinin[4] in the amniotic or allantoic liquids.

Cell cultures

Tissue culture has long been used in laboratories since the impetus given to its use by Carrel, but it remained an extremely delicate technique because of the difficulty of supplying the cells with a suitable medium,

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animals were not shown to be sensitive. In 1933, Smith, Andrewes and Laidlaw, having found that the ferret was capable of being infected naturally or experimentally by a virus of the dog, had the idea of trying to transmit influenza to the ferret. They showed that this transmission was possible. Two days after inoculating the ferret intranasally with the liquid from the throat-wash of a man with influenza, the ferret developed signs of influenza: depression, watering of the eyes, a running nose and sneezing: the ferret recovered in about ten days. But it transmitted the disease to another ferret placed near it and its lungs contained, at the time when its temperature was high, a large quantity of virus that could be similarly used to infect other animals. The same research team also showed that the lungs of an infected ferret contained a virus equally virulent for white mice, thus demonstrating a further simplification of technique. Although the mouse, one of the most useful of laboratory animals, was not sensitive to the virus coming directly from man, it could be infected without much difficulty with the ferret-passaged virus and very regularly with virus passed through mice and 'adapted' to this host.

(4) Haemagglutinin: *a substance which agglutinates red blood cells.*

(5) Trypsin: *an enzyme, present in pancreatic juice, which digests albuminous matter.*

(6) Strain: *this term is used in the sense of a continuously subcultured line. It is applied to a virus or to living cells which are cultivated by successive passages.*

(7) *It has also been possible to isolate strains of stable cells from human tissues which are non-cancerous (amnion, skin, liver, conjunctiva, appendix, etc) and from healthy animal tissues. These strains of cells are maintained today like strains of bacteria. They can be used*

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either for isolating a virus or for measuring the quantity of a virus; that is to say, for making a titration or for maintaining and studying a virus. These methods are used for the study of certain viruses for which a convenient animal does not exist.

(8) *Viruses measure from 15 to 300 mμ (millionths of a millimetre). This means that in a 1,000,000 times enlargement, where a human red blood cell appears as a disc 7 metres in diameter, the poliomyelitis virus would appear as a marble of 2 cm diameter and the smallpox virus as a cube with 30 cm sides.*

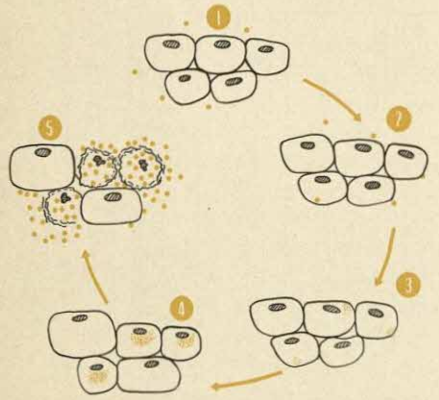


Fig. 2. Representation of the multiplication of a virus in sensitive cells. (1) The virus is brought into the neighbourhood of sensitive cells; (2) the virus particles attach themselves to the cell membranes and penetrate the cells; (3) the virus which has penetrated disappears; it is not possible either to see or recover it by destroying the cells: it is an 'eclipse' phase; (4) the virus reproduces in the cell, to the detriment of the normal functioning of the cell. The cell shows signs of suspense; (5) the infected cells liberate the virus which can then infect new cells; the multiplication cycle is thus completed.

and above all because of contamination by airborne microbes. The use of antibiotics, which are for the most part inactive against viruses, has led indirectly to a great advance in virology by rendering the culture of living cells much less hazardous.

Progress has also been made in refining the media used for culture and by using trypsin[5] to break down tissues. In this way suspensions of cells in a nutritive medium containing antibiotics can be readily obtained. These are set up for culture in glass bottles. The cells attach themselves to the glass and it suffices to change the medium at regular intervals to keep them in a healthy condition and obtain active multiplication. By suitably choosing the animal and the organ to effect the trypsin digestion, cultures are obtained of cells which are sensitive to the virus that is to be studied (fig. 2).

Two types of cell culture exist: normal and transformed. A normal culture is one where the cells are obtained directly by digestion of an organ, placed in culture and inoculated directly, or, strictly, after transfer to a second flask with a further trypsin treatment. If, however, several successive transfers are made, it sometimes leads to a transformation of the cells: they become more uniform but also more active and reproduce more abundantly. Their number increases rapidly and they crowd the flasks. These cultures can be indefinitely subcultured to conserve the strain[6] of the cells. This transformation was first used successfully with human cells of cancerous origin, such as the strains 'Hela' and 'ICB', well known throughout the world, which were isolated in 1953 and have been maintained ever since.[7]

The disadvantage of using chick embryos and tissue cultures lies in the difficulty experienced in identifying the virus. With animal inoculation, identification by the observation of recognizable illness is possible. One can see by other signs that the virus has multiplied: the death or stoppage of growth of the embryos or the degeneration of cells in culture. This degeneration marks the pathogenic activity of the virus for sensitive cells, its 'cytopathogenic' effect. The following very neat method, worked out by Dulbecco in 1952, allows the cytopathogenic effect to be measured. A culture of cells, infected and fixed to the walls of a flask, is immobilized by the pouring through it of a thin layer of a medium which can solidify (gelose medium). Spots or plaques of cell destruction become visible, each one corresponding to a unit of virulence. It suffices to count these spots to obtain a measure (or titration) of the virus in the inoculation.

How then do we class in such or such a group a virus which has been isolated? It is relatively easy when one already knows the properties of the virus to which it is thought possible to connect the strain that is being studied: its experimental pathogenic properties, such as the production of specific symptoms in a laboratory animal, or its biological properties, such as the capacity which certain viruses have of agglutinating red blood cells, or the production of specific lesions in the cells of organs of inoculated animals or in certain tissue-culture cells.

Immunological identification

In the majority of cases, the method of immunological identification makes use of the very highly specific characteristics of the phenomenon of immunity. When an individual has been subjected to a virus disease and has recovered, he is usually no longer sensitive to it; he is in fact immune. This state of immunity remains for varying lengths of time, depending on the individual and on the virus. In some cases it is permanent, in others it disappears after several months or years, but in any event it is specific for the virus which caused the disease. A convalescent from mumps cannot be attacked by the virus of the disease again, but he is still as sensitive as before to other viruses. This state of immunity, natural or promoted experimentally in laboratory animals, can be used for the identification of an unknown virus.

Let us take as an example the poliomyelitis virus, of which there are three distinct types (I, II and III). Suppose we wish to identify a virus isolated and maintained in tissue culture and have reason to suppose that it is one of the viruses of poliomyelitis. We inoculate this virus into four monkeys, one immunized against type I, another against type II, another against type III, and the last not immunized at all. If, for example, the non-immunized monkey and the monkeys immunized against types II and III show typical signs of poliomyelitis and die, and the remaining monkey immunized against type I stays alive and healthy, we can conclude that the virus was of type I. A counter-test can be made by vaccinating another monkey with a vaccine prepared from the unknown virus and then inoculating it with the virus of type I. The monkey should survive, but remain sensitive to inoculation with a dose of, for example, the virus of type II.

Serology

These immunological tests are laborious, expensive and time-consuming. Fortunately they have been greatly simplified by using serum antibodies. One of the mechanisms of immunity is the production of specific substances in the serum of the blood, the antibodies, which combine with the agent responsible for the attack—in this instance a virus—and neutralize its pathogenic activity.

This specific combination of virus with antibody can be shown in several ways. The simplest consists of mixing the virus with a virus-serum; if antibodies are present they will combine with the virus, thus neutralizing its pathogenic properties, so that inoculation of this mixture into a sensitive animal will not produce symptoms. Other methods, such as the precipitation reaction, the complement-fixation reaction and the inhibition of haemagglutination, allow the same combination to be shown. The method of action is diverse, but all the methods are aimed at tracing the specificity of the combination between virus and antibody where one of the two constituents, virus or antibody, is known in advance, and the other as yet unknown.

These so-called serological methods are widely used in the diagnosis of virus diseases in man. It is no longer necessary to isolate the virus in order to identify the disease: it is simpler to identify instead the antibodies in the serum of the patient. The most appropriate methods for each virus are now in general use and many diseases can be definitely diagnosed; for example, by using the complement-fixation reaction for the influenza virus and the neutralizing reaction for the poliomyelitis virus.

Microscopic observation

This outline of the techniques used in virology shows that direct observation plays only a minor part. The small size of the viruses renders the study of their morphology impracticable for general use. In order to see the virus particles, it is necessary to use an electron microscope, which shows images that are fairly often difficult to interpret in detail and which give above all an idea of the size and form of the virus[8] (fig. 3).

Viruses of human and animal diseases appear as spheres (or sometimes as cubes) with diameters varying from 15 to 300 millionths of a millimetre. But viruses cannot be identified by their electron microscopic images. Indeed, it is more interesting to observe the cells in which the virus is multiplying. This multiplication, which most often ends with the cell's death, at first causes damage in the cellular functions, certain aspects of which can be watched under the microscope, using techniques such as dark-ground or phase-contrast microscopy or by specific histological and cytological colour staining.[9]

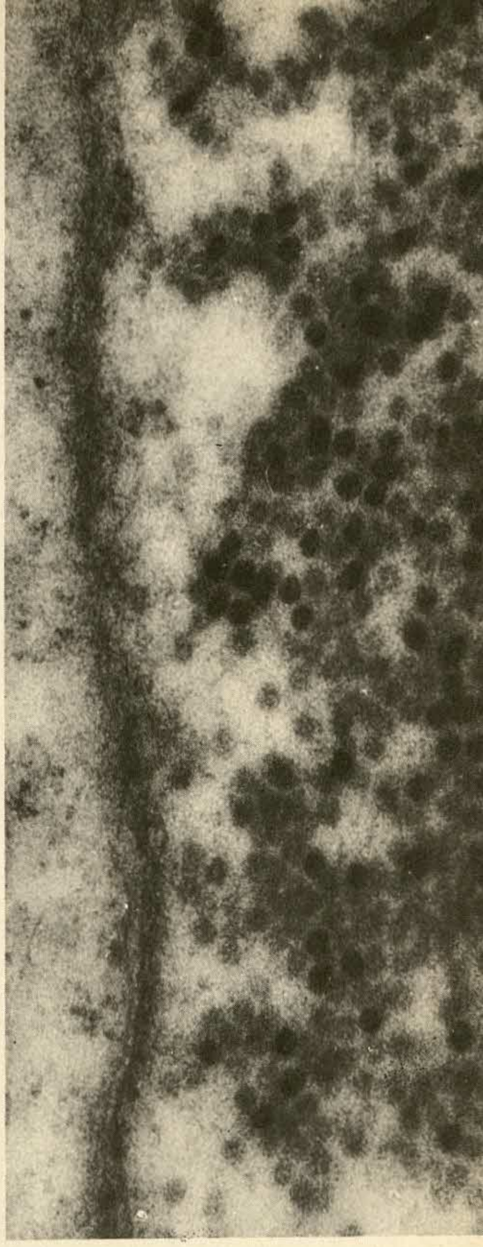


Fig. 3. An electron microscopic graph of cells within which mouse polyoma viruses are multiplying, sometimes causing a cancer. (Mag. × 88,800. Electron micrograph by Dr W. Bernhard of the Institute of Cancer Research, Villejuif, France).

These methods were the only ones available when virology was a young science and were consequently very important. Recent technical progress has reduced their interest, although certain of them remain indispensable.

Conservation of viruses

It is not always essential, in order to maintain a virus strain in the laboratory, to pass it without interruption from one animal or culture to another. Normally, viruses can be preserved by freezing at low or very low temperatures (−25°C, −70°C or even −150°C), or by desiccation or lyophilization.[10] Such preparations retain their 'viability' for days, months or years, depending on the type of virus. It is very important to be able to conserve viruses, not only for theoretical study on their pathogenic properties, variation, sensitivity to medications, etc, but also for the preparation of vaccines. Most viruses are insensitive to antibiotics, so the only measures that it is possible to take against them are preventive. Vaccines are now made against a number of viruses (smallpox, rabies, poliomyelitis, yellow fever, influenza, mumps and glandular fever) and some of them have caused the retreat, if not the disappearance, of dangerous diseases.

One is filled with admiration at advances made in virology in the last few decades: from the original outstanding discoveries of the agents responsible for the most dangerous diseases in man and animals, to the perfecting of more and more ingenious and complex laboratory techniques which have led to the understanding of some of the essential properties of viruses, and finally to the development of methods of combating viral diseases. There are few examples in biology of disciplines where so much progress has been made so rapidly.

(9) *Some viruses cause, under well-defined conditions, the appearance of characteristic intracellular lesions. For example, in some cells inclusions arise which are of abnormal colour, and can be recognized immediately as being due to a particular virus. These inclusions have long been known for several diseases: the Negri bodies, in cerebral cells of animals suffering from rabies (their presence in the brain of dogs is still the quickest method of diagnosing this disease), the Guarnieri bodies of the vaccinia virus, Councilman bodies in yellow fever, and many others.*

(10) Lyophilization: *a method whereby a substance previously frozen can be dried. This method damages the biological products less than does ordinary desiccation.*

Viruses and cancer

Léon DMOCHOWSKI

What is the cause of cancer in human beings? The answer is not yet known, but there is hope that it will be found before long. Indeed, combined efforts in research have brought considerable progress in our knowledge of nearly-allied cancers, namely those in animals. Various factors can play a part in the origin of animal cancers: a certain genetic constitution, hormonal disorders, metabolic disturbances, varying chemical substances—and viruses. The role of viruses, without necessarily being of prime importance, appears nevertheless to be extensive. A thousand facts prove this, from those discovered by Peyton Rous, towards the end of the nineteenth century, to those very recently revealed by the study of the extraordinary polyoma virus. That these viruses have an action in the origin of human cancer also is the opinion of the best minds of our day.

Cancer[1] is a disease which affects plants and animals, as well as human beings of all races and ages. It is not a disease of civilization, though its occurrence is more frequent nowadays than in previous times because prevention and treatment of infectious diseases has led to a prolongation of the average life-span. Although no age group is free of cancer, it does occur more often in later age.

Definition of cancer

Cancer is a popular term for a group of diseases which develop in all organs and tissues. The terms 'benign' and 'malignant' are an arbitrary distinction between so-called benign and malignant tumours induced by viruses. There is no sharp distinction between normal and tumour cells, whether benign or malignant. A gradual change appears to take place, step by step, from a well-organized cellular pattern to the unrestricted behaviour of cells in a malignant tumour, or cancer, followed by the invasion of surrounding and then of distant normal tissues and their final destruction.

Any type of tumour is produced by the most diverse factors, some of which are known, but many of which still remain to be discovered. The factors known to play a part in the origin of tumours include genetic constitution, hormones, metabolism, viruses and such environmental factors as various chemical compounds, and radiation energy. No type of tumour, as such, is inherited, only susceptibility to the disease is inherited; other factors such as those already mentioned must be present before a tumour finally develops.

Micro-organisms are to be found everywhere. They will colonize any environment—warm springs, sulphur deposits, and minerals, as shown in these stones of Versailles, eroded by bacteria and mosses living on the surface.

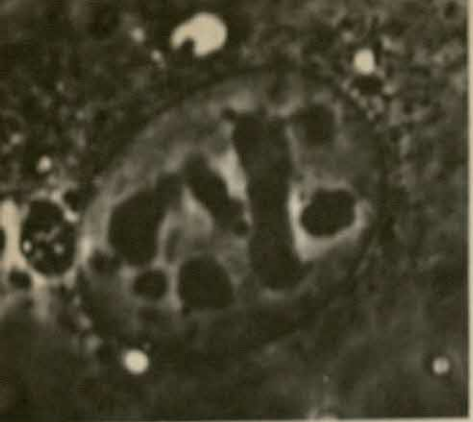


Fig. 1. Living cell of mouse embryo with polyoma: note the development of nucleoles and nuclear inclusions. (Phase contrast microphotograph, mag. × 1,400, after E. Bereczky and L. Dmochowski).

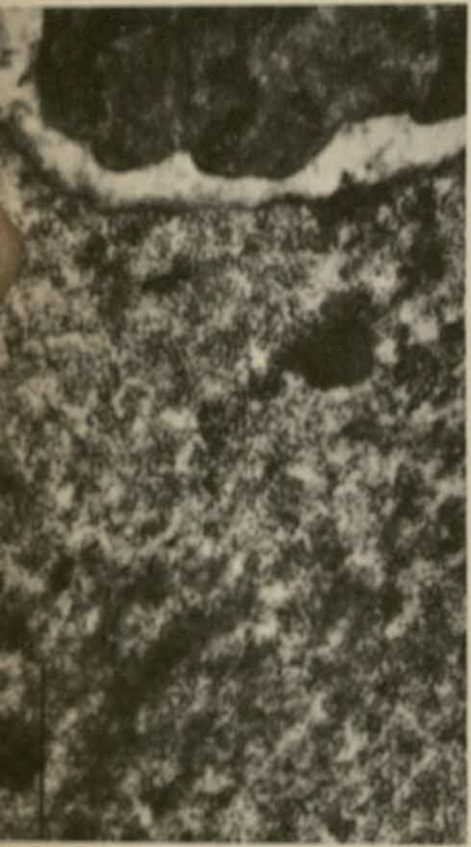


Fig. 2. Kidney tumour in a hamster, following a polyoma. Numerous viral particles are to be seen in part of the nucleus (above) and in the cytoplasm (below), where they are regularly arranged. (Electron microphotograph, mag. × 25,000, after L. Dmochowski, E. Bereczky and C. E. Grey).

Definition of viruses

Viruses are nucleoproteins, bodies composed of proteins and ribonucleic[2] or deoxyribonucleic acid,[3] which reproduce only within cells from the cells in the nucleic acids, and are devoid of enzymes.[4] They are capable of entry into susceptible cells in which they reproduce similar entities, and are therefore described as infectious. During reproduction, they may produce symptoms of a disease or a tumour. They may remain latent or dormant for long periods of time. This point is of special importance in tumours. The cells of a susceptible host may or may not show visible signs of infection by a virus; they may show different changes or symptoms according to the age of the host, the amount of the virus and the route of its entry.

Some viruses may produce inflammatory, suppurative, necrotizing, and therefore destructive, lesions in the infected host; the same or other viruses may lead to anarchical cellular proliferation, ie cancerous lesions. The former classification of viruses into 'infectious' and 'tumour' viruses was misleading, as studies have failed to reveal any essential differences between these two categories.

There is an impressive similarity between the advances made during the last ten years in the studies of viruses as causative agents of infectious diseases in man, and in those on the origin of tumours in animals. More than 150 new viruses responsible for infectious diseases in animals and man have recently been described. During the same period of time, a number of viruses have been found to be responsible for various types of tumours in animals.

Electron microscopy has revealed an essential similarity in the sub-microscopic structure of cells of plants, animals and man, and in the morphological structure of plant, animal and human viruses. Biochemical studies have demonstrated that viruses of plants, animals and man contain a core of nucleic acid, surrounded by a coat of protein. Among other properties common to tumour viruses and other viruses, is their ability to remain latent or quiescent for long periods of time. They may be transmitted in a latent manner either through the ovum or sperm, or through mothers' milk, from generation to generation of animals, and appear suddenly as a 'spontaneous' disease. They may also be spread by particles of dust, by food, drinking water or direct contact.

Tumour viruses

It is known that viruses cause tumours in plants, invertebrates and vertebrates. Many types of tumours of different organs and tissues of animals are induced by viruses, but there is as yet no experimental proof of any one type of human malignant tumour or cancer being caused by a virus. It is, however, difficult to believe that there exists a sharp distinction between cancer in animals and in man. It may not be long before present-day studies, combining the efforts of many specialists in the various branches of science, reveal the possible relationship of viruses to at least some types of cancer, such as leukaemia, in man.

Long before the first tumour of viral origin in chickens was discovered by Ellermann and Bang, Borrell and Bosc advanced the hypothesis of viruses being responsible for cancer. After the discovery of the viral origin of sarcoma in chickens by Peyton Rous, and its confirmation, as well as the discovery by German, English, Japanese, American and Italian workers of other tumours in chickens caused by viruses, the idea gradually gained ground that tumours in birds may be in a different category from those in mammals. Studies which followed revealed that at least some tumours of mammals are caused by viruses. The discovery by Shope of the viral origin of fibroma[5] and papilloma[6] of rabbits broke down this artificial distinction. Nevertheless, proof of the viral origin of a true 'malignant' cancer was lacking. This was provided by Bittner's discovery of the viral origin of mammary cancer in mice of genetically homozygous or pure strains, after more than thirty years of studies which would not have been possible without the development of inbred strains of mice, originally introduced by Little. The discovery was all the more important because it led to the realization of other factors, such as the age and susceptibility of the host in the development of breast cancer in mice, a discovery that has helped to remove all doubt concerning the implication of a virus in the origin of a malignant tumour.

At about the same time, a most interesting biological phenomenon was described in the transformation of a live fibroma virus of rabbits by heat-inactivated myxoma virus into a live myxoma virus, which causes a fatal infectious disease in rabbits. It is now known that the deoxyribonucleic acid of the myxoma virus is responsible for this transformation of a tumour virus into one causing infectious disease.

Another milestone was the discovery by Lucke of the viral origin of a malignant tumour in frogs. Thus, evidence was accumulating of viruses playing a part in the origin of malignant tumours not only in birds and mammals but also in cold-blooded animals.

About the same time, Duran-Reynals showed that the virus of Rous sarcoma of chickens may induce a destructive disease in young chicks and ducklings, and many types of tumours in older birds of other species. It was astonishing that one virus could be responsible for all these diseases. Many years were to elapse before a similar observation was reported in mice by Stewart and Eddy. Meanwhile, Duran-Reynals reported that the virus of a benign Shope fibroma of rabbits may, under certain conditions, cause either a lethal inflammatory disease or malignant tumours in rabbits.

Gross was the first to demonstrate the viral origin of a lymphatic leukaemia in mice, associated with over-activity of lymphoid tissues and the presence of young lymphocytes in circulating blood. Leukaemia is a term for neoplasms of the blood and blood-forming tissues, such as granulocytes, lymphocytes, reticulum cells, stem cells and others. The use of new-born mice of certain inbred strains led to the discovery by Gross of the viral origin of lymphatic leukaemia in mice. Gross also observed the development of cancers of the salivary gland and adrenal glands and of sarcomas in mice injected with the virus of lymphatic leukaemia.

This gave a great impetus to studies on the viral origin of leukaemia in animals. Another type of leukaemia in mice, myeloid chloroleukaemia, affecting myelocytes and young white blood corpuscles, was also found,

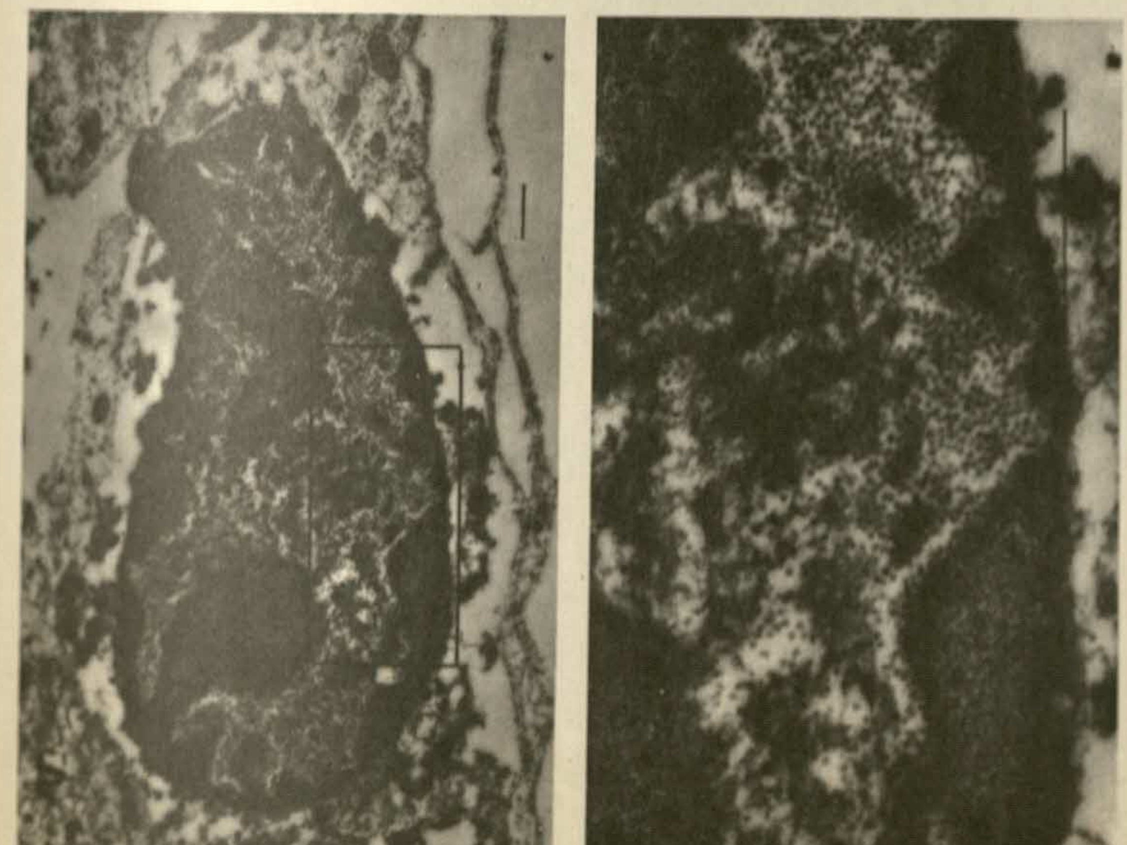


Fig. 3. The presence of virus particles in living cells causes damage to the cytoplasm and nucleus. The metabolism of the cell and the process of cellular division often show characteristic changes. Here, a mouse embryo cell infected by the polyoma virus is cultivated in an artificial medium. The nucleus contains chromatin as well as many virus particles. The cytoplasm shows signs of degeneration (mag. × 8,400). Fig. 4. On the right, greater enlargement shows the polyoma virus particles, closely associated with the chromatin, and the thickening of the nuclear membrane. (Mag. × 32,000, after L. Dmochowski, E. Bereczky and C. E. Grey).

(1) Cancer *is the term usually applied to any malignant tumour. Pathologists use the term epithelioma for tumours which originate from epithelial tissues, and sarcoma for tumours of connective tissue. Tumour or neoplasm are names used for any independently growing cells, whether malignant or benign.*

(2) Ribonucleic acid *is a compound originally found in yeast and later in the cytoplasm and cellular nucleoles.*

(3) Deoxyribonucleic acid *is a compound originally found in the thymus gland and later in the chromatin of cell nuclei.*

(4) Enzymes *are organic compounds which accelerate or produce by catalytic action changes in the substrate, upon which they exercise a generally specific action.*

(5) Fibroma *is a tumour composed mainly of fibrous or fully developed connective tissue.*

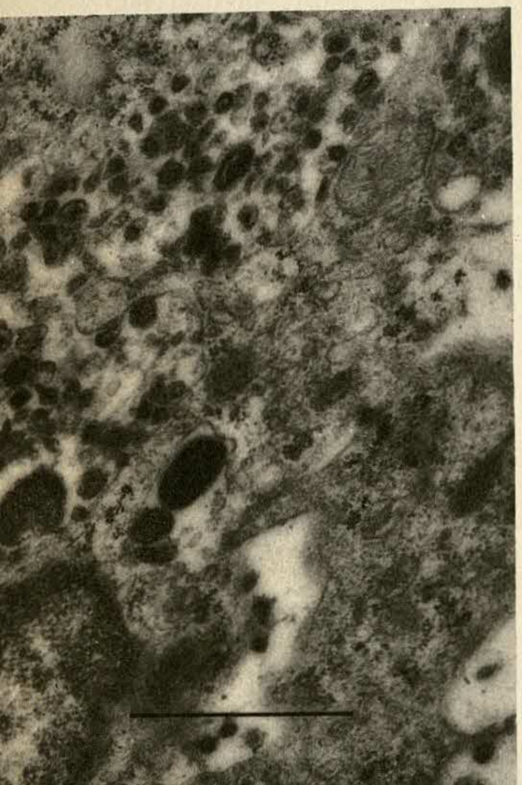
(6) Papilloma *is a tumour of epithelial tissue in which the cells cover finger-like processes or ridges of stroma (supporting tissue).*

by Graffi and his associates, to be of viral origin. They observed that this virus will induce a similar type of leukaemia in rats and, transferred back from rats to mice, will give rise to the same disease, as well as others, such as lymphatic and reticulum cell leukaemia. The relationship of Gross's virus to that of Graffi is unknown. The number of leukaemias of viral origin in animals is rapidly rising and the question of the possible viral origin of leukaemia in man has become of pressing interest. There are great similarities between infections and leukaemia in man, both in the onset and in the course of the disease.

Studies on the lymphatic leukaemia virus of Gross have led to the isolation of a most interesting tumour virus by Stewart and Eddy through the use of tissue culture of mouse embryo cells on which extracts of leukaemic tissues of mice were passaged. Material from such cultures was found to induce in mice an astonishing multiplicity of tumours of different tissues and organs anaemia, dwarfism, cataract, inflammatory and destructive lesions, frequently many in one animal. In addition, the virus was found to induce many types of tumours in rats, hamsters and rabbits. The virus, described as polyoma, or a multiple tumour-inducing agent, induces antibodies in animals of the different species it infects, and its spread has been investigated by means of immunological tests and its presence observed in inbred mice and wild mice. Successful attempts at vaccination against this virus have been reported. Immunization against lymphomatosis virus in chickens has also been reported.

Leukaemia has been accelerated in mice of strains with a high incidence of leukaemia, and induced in mice of strains with a low incidence of the disease, by means of cell-free filtrates of brains from leukaemic mice and of brains from people who have died from leukaemia. This accelerating and leukaemia-inducing agent has all the properties of a virus, but the relationship of the agent in the brains of leukaemic mice to that in the brains of leukaemic humans is unknown.

The number of tumour viruses is constantly increasing, but there is no doubt that one tumour virus may cause different types of tumours, and similar types of tumours may be caused by different viruses.



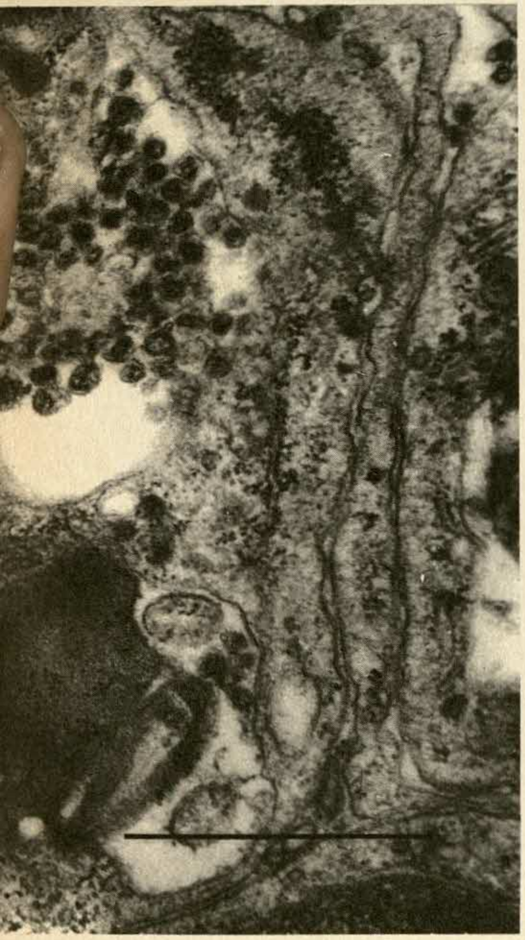


Fig. 5. General appearance of lymph node in a patient with acute lymphatic leukaemia. The virus particles are centred in the picture (mag. × 28,000). Fig. 6. The virus particles in greater enlargement, shown in a vacuole of the same cells (mag. × 38,000).

Viruses and other cancrigenic factors

Without inherited susceptibility and suitable hormonal factors, breast cancer in mice will not develop, in spite of the presence of the mammary tumour virus. On the other hand, breast cancer may develop in mice which have a suitable genetic constitution and hormonal make-up, and are apparently virus-free. Genetic factors play an important part in the development of Rous sarcoma and lymphomatosis in chickens. The age of the host is an important factor; with certain exceptions, the younger the animal the more it is susceptible to tumour viruses, although the end result of the infection, a tumour, usually (polyoma virus excepted) does not become evident until middle or old age. Hormones, radiation and the thymus gland, have an influence on lymphatic leukaemia in mice. Hormones may enhance or inhibit the diseases, while removal of the thymus gland prevents or decreases it. Cell-free extracts of mice tissues with leukaemia induced by x-rays have produced it in healthy mice, and treatment of certain mice with cortisone leads to the development of tumours of viral origin. These experiments have shown that some tumour viruses may exist in animals in a latent form and may be transmitted from generation to generation without inducing tumours. They are non-infectious in the ordinary sense, but under certain circumstances regain their activity. Removal of the thymus in mice inoculated with the Gross virus results in the appearance of a different type of leukaemia (myeloid). This raises the problem of whether the same or a different virus is involved.

Electron microscopy studies of tumours and tumour viruses

Electron microscopy has demonstrated a basic similarity in the appearance and internal structure of plant, animal and human cells, and of bacterial, plant, animal and human viruses, whether seen within the infected cells or in an isolated state. Viruses may vary in size, shape and some details of internal structure, but all show a basic structure composed of one or more protein membranes surrounding a core of ribonucleic or deoxyribonucleic acid. The characteristic structure of virus particles differentiates them from normal cell components. Electron microscopy of tumour cells has therefore been of fundamental importance in the search for virus particles.

The electron microscope has, however, so far failed to reveal any specific or qualitative difference between the sub-microscopic structure of normal and tumour cells. The only difference is the presence of virus particles in the cells of tumours of viral origin, but it is not always easy to find virus particles, even in tumours induced by viruses, an important point in any future studies of human tumours. The importance of electron microscope studies of tumours induced by viruses lies in their demonstration of the causative agents of these tumours as morphological entities.

One of the first tumours to be examined in thin sections in the electron microscope was breast cancer of mice. Characteristic virus particles were found in the cytoplasm of tumour cells and outside the cells; electron microscopy combined with biochemical, biophysical and biological techniques show that these particles are indeed the causative agent of breast cancer in mice.

Although the search for virus particles in the circulating blood cells of chickens with fowl leukaemia (lymphomatosis, erythroblastosis, myeloblastosis) has proved mostly unsuccessful; the same cells grown in tissue culture revealed virus particles with comparative ease. Again, electron microscopy dispelled any doubts as to the nature of these virus particles. The morphological similarity of virus particles found in all forms of chicken leukaemia is indeed striking, and it raises the question of whether the same virus is responsible for them all. The virus of chicken myeloblastosis (characterized by the presence of myeloblasts or young white blood cells in the circulating blood) is known to induce cancer of the kidneys in chickens. Electron microscopy of this kidney tumour has revealed virus particles morphologically identical with those observed in myeloblastosis.

It took many years before virus particles were discovered in the Rous sarcoma and proved to be the causative agent of this tumour, known to be of viral origin for almost fifty years.

Most recent studies of myeloid leukaemia, induced by the virus of lymphatic leukaemia in mice after removal of the thymus gland, have revealed particles morphologically similar to those found in lymphatic leukaemia. Thus, suggestive evidence is available that the same virus may be responsible for both types of leukaemia in mice. All types of leukaemia in mice, found to be of viral origin, have now been examined under the electron microscope and have revealed the presence of virus particles of characteristic size and shape.

Electron microscopy has also led to recognition of the virus of Shope fibroma and papilloma of rabbits. Tissue culture studies combined with electron microscopy and biological tests, demonstrated that these viruses are the specific causative agents of these tumours.

Studies of carcinoma of frogs' kidneys have revealed the presence of virus particles, both in the nucleus and cytoplasm of tumour cells, similar in general appearance to those observed in tumours of chickens, mice and rabbits.

The discovery of the polyoma or multiple tumour-inducing virus in mice, rats and hamsters, immediately raised the problem of whether one or more viruses are involved in the origin of these tumours. Appropriate biological experiments have indicated that one virus appears to be the causal agent of all the lesions and tumours of diverse types, but this still requires morphological confirmation. Electron microscope studies of cells grown in tissue culture and infected with polyoma virus have now shown characteristic virus particles in the nuclei and also in the cytoplasm of these cells. Thus, electron microscopy proved the viral nature of changes observed by phase microscopy in living polyoma-infected cells, and by fluorescence microscopy in cells stained with fluorescent dyes. Morphologically identical virus particles were also found in the kidneys of polyoma-infected mice, rats and hamsters. Electron microscope studies have therefore confirmed that a virus may act not only as the initiating but also as the continuing cause of malignancy.

The progress in electron microscope studies of animal tumours led to similar studies of human tumours, especially leukaemia, long suspected to be of viral origin. Studies of lymph nodes obtained by surgical operations from patients with different types of leukaemia have revealed changes similar to those found in mouse and chicken leukaemia, and also virus particles in half of the cases examined. Tissue culture studies have shown changes in the cells suggestive of the presence of a virus, and electron microscopy, the presence of virus particles within these changes. Similar changes or virus particles could not be found in the cells from lymph nodes of patients apparently free of leukaemia.

Although these studies do not indicate that the virus particles are the causative agent of leukaemia, nevertheless they are a preliminary and indispensable step in the studies on the viral origin of human leukaemia and other tumours.

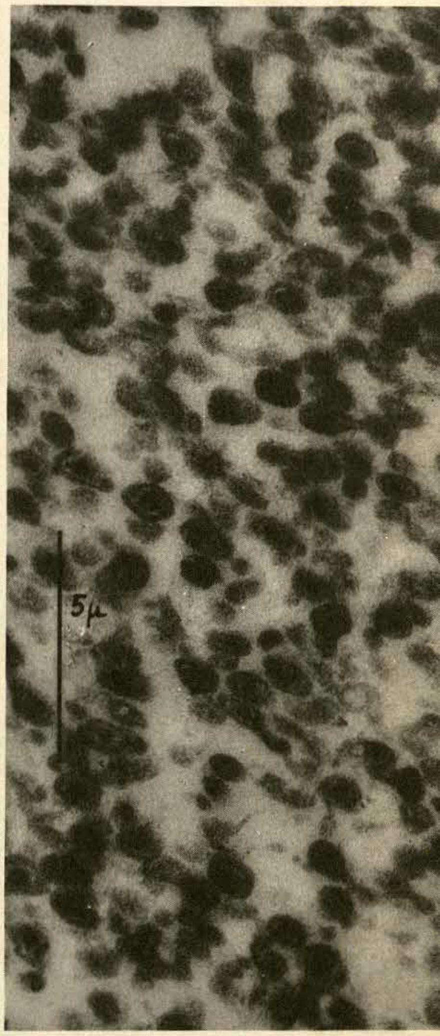
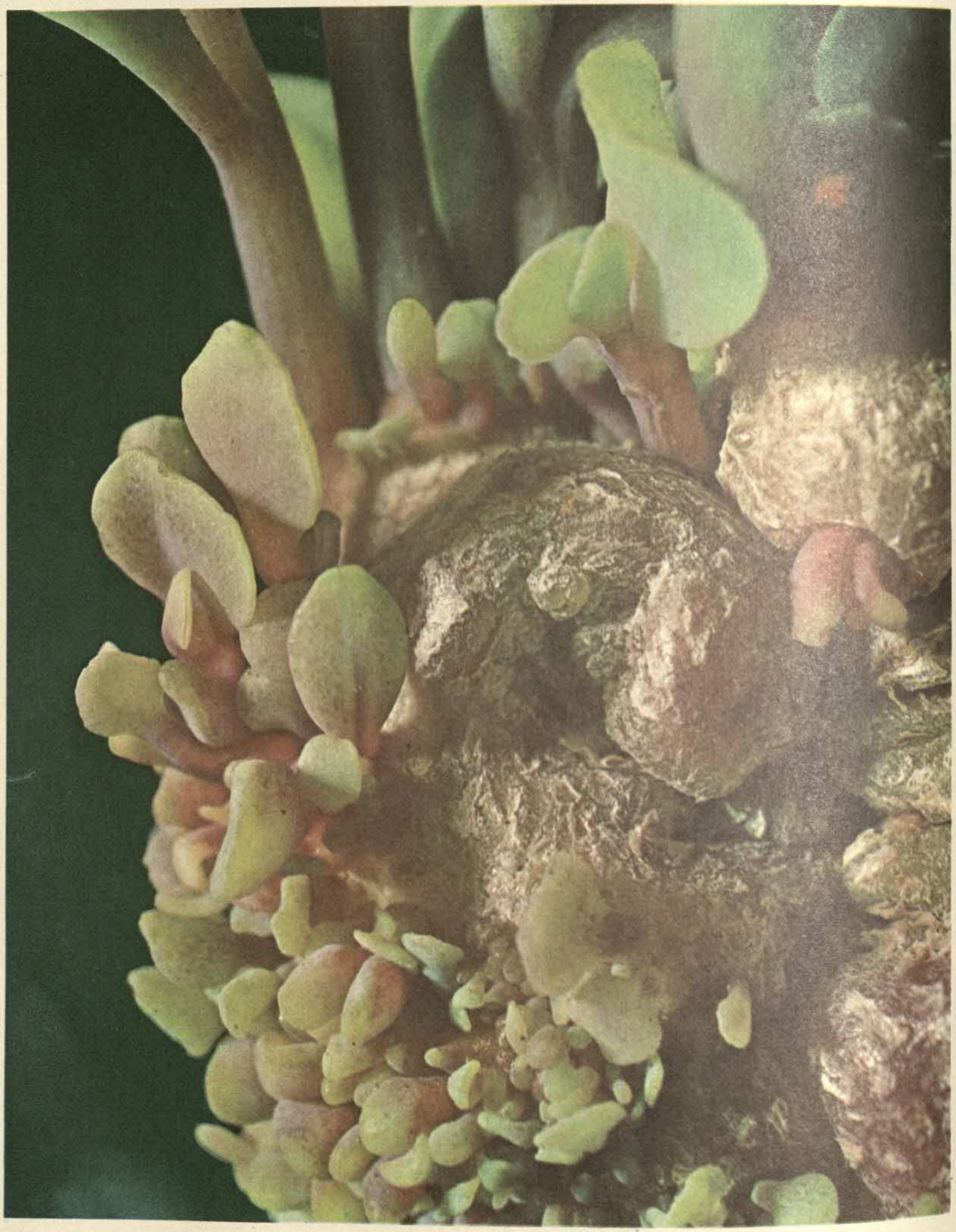


Fig. 7. A mass of virus particles obtained by slow- and high-speed centrifugation of milk from mice with mammary tumours. Fat and casein have been removed and the milk treated with fluorocarbon (magnified 60,000 times, L. Dmochowski and C. E. Grey).

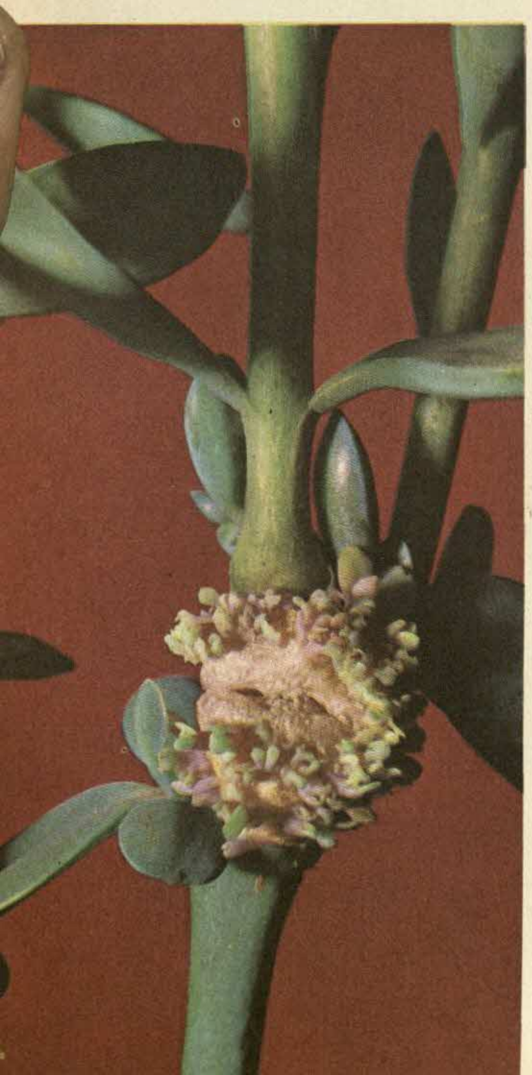
Plant cancers

Experimental production of tumours is one sure means of identifying bacteria: the form and variety of colouring give valuable information. The tumours shown here have been obtained by the injection of Agrobacterium tumefaciens *(see page 60)*.

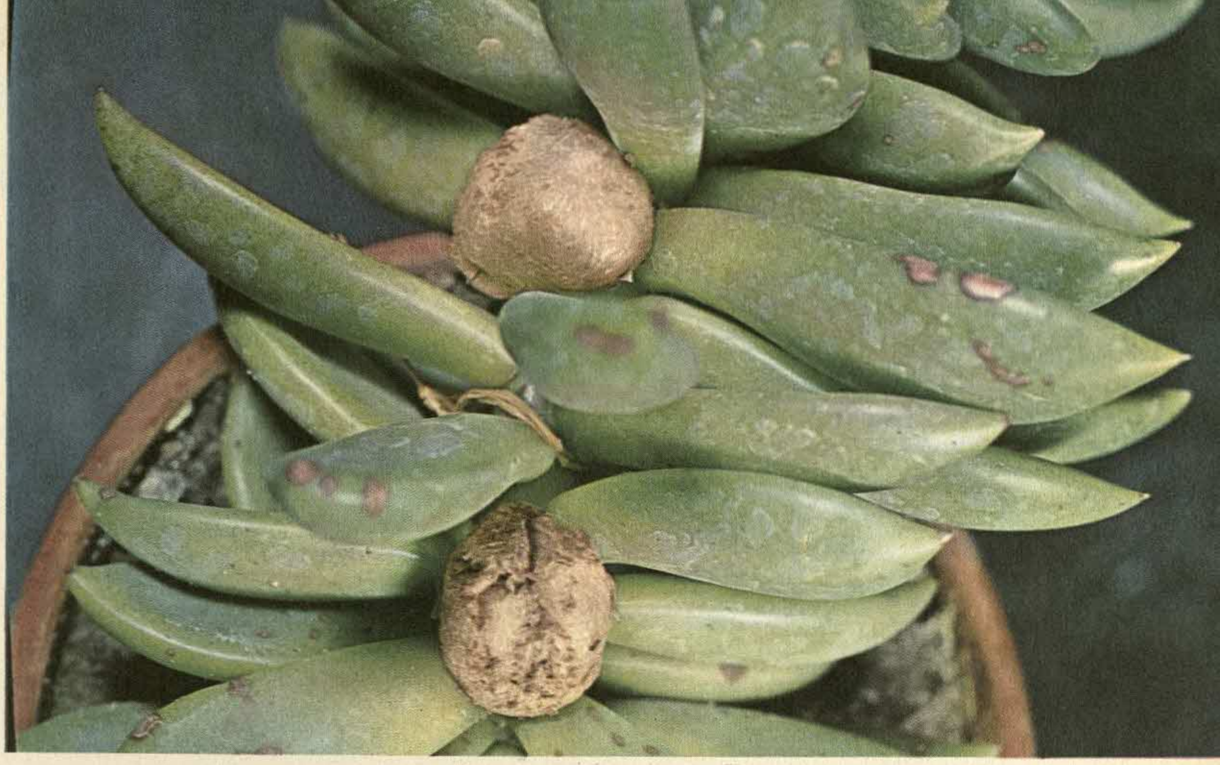
A tumour greatly magnified (right). Besides the leaf shoots which are still visible, a great variety of pigments have been obtained.



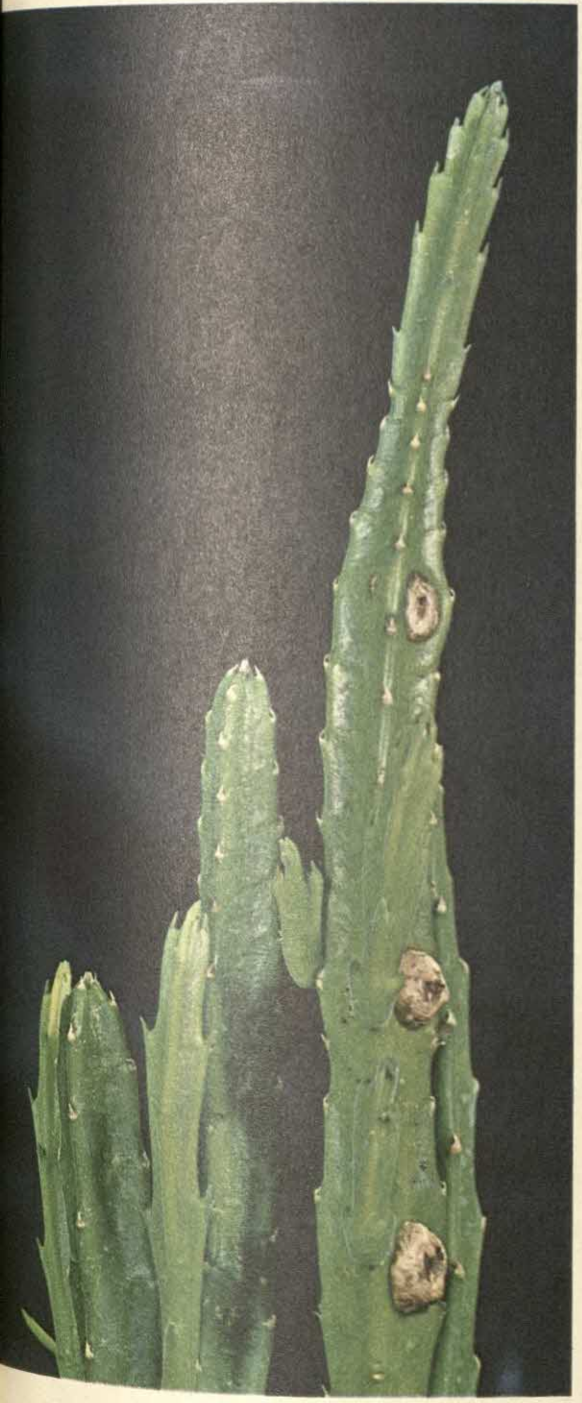
Some bacteria, including Agrobacterium tumefaciens, *below, can induce tumours in a variety of plants. An external tumour obtained on Kalanchoe rotundifolia has produced numerous leaf shoots.*



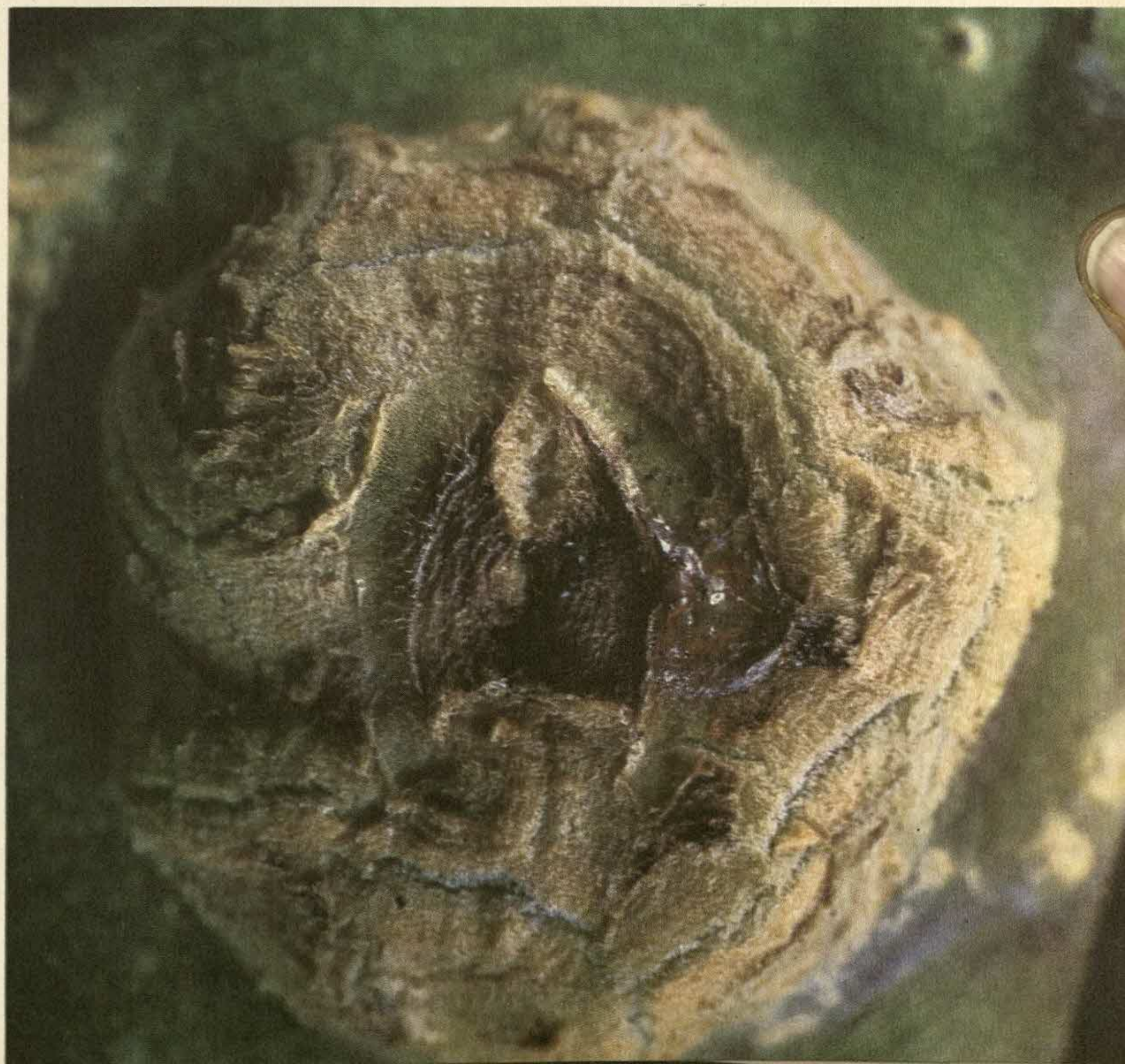
Right. Inoculation of the plant Stapellia grandiflora *produces greyish clumps. Abnormal shoots, lighter in colour than the normal shoots, are formed close to the tumours.*



On Echeveria scaphophylla *(left) inoculation produces several tumours on the same shoot.*



The middle tumour of the preceding plant, greatly enlarged. The circular arrangement of the cancerous tissues is very marked. (Photo Lod, Laboratory of J. G. Marchal).



The bacteriophages

Pierre NICOLLE

Bacteriophages (the word is now generally abbreviated to phages) are viruses capable of attacking and destroying numerous species of bacteria. The bacteriophagic phenomenon, discovered by Twort, and afterwards intensively studied by d'Hérelle, is of the greatest interest to microbiologists. There was a time when bacteriophages were thought to play at least as great a role in the processes of antibacterial immunity as that played by phagocytes. There was also a time when it was thought that they could be used therapeutically. These times have for the most part passed. Instead, the use of bacteriophages has become one of the commonest methods used in the precise identification of certain strains of bacteria. Above all, the intensive study of the properties of bacteriophage particles has added much to our knowledge of the major processes in living matter.

The bacteriophagic phenomenon has been the object of very many theoretical and practical studies, particularly during the last twenty years. The bacteriophages are, in fact, much more accessible for detailed research and experiment than most viruses,[1] and the results obtained are, to a certain extent, of general application.

It has been known for a long time, through ultrafiltration,[2] ultracentrifugation[3] and various other methods, that bacteriophages are composed of elementary corpuscles which vary in diameter, depending on the type, between 20 to 100 millionths of a millimetre.

With analytical processes which are now very much improved, such as the electron microscope,[4] electrophoresis,[5] chromatography,[6] serology,[7] immunochemistry,[8] and particularly the use of radioactive isotopes,[9] great progress has been achieved in our knowledge of bacteriophage corpuscles. A phage has a polyhedral head and a tail of varying length and thickness formed from an axial cylinder surrounded by a kind of tube (figs. 3 and 4).

Each corpuscle is composed principally of two substances very different in their chemical nature and their physiological properties. The envelope which surrounds the head and extends along the tail is of protein and the small central mass, which can be thought of as a nucleus, is composed almost exclusively of deoxyribonucleic acid.[10]

The protein envelope functions in the first place as a protective coat for the deoxyribonucleic acid, which is the active but delicate part of the virus, protecting it from destructive influences in the medium, particularly the action of certain bacterial enzymes[11] such as deoxyribonuclease. Moreover, it keeps back for itself the specific antigenic properties[12] of each variety of bacteriophage. When a bacteriophage is injected into a rabbit, there appear in the animal's serum substances called 'antibodies' which are capable of combining with the bacteriophage that induced the formation and neutralizing it, that is, rendering it inactive; but these substances do not act against other bacteriophages.

The antiphagic sera, because of their specific neutralizing properties,[12] are very much used for the identification and classification of bacteriophages.

At the free extremity of the tail the protein envelope has two very important functions: (1) it has the power of attachment to

(1) Virus: *see the chapter entitled 'What is a virus?'*

(2) Ultrafiltration: *filtration through membranes of collodion, cellophane, etc. By means of experiments with membranes of different pore sizes it has been possible to deduce the dimensions of virus particles.*

(3) Ultracentrifugation: *centrifugation at very high speeds. The speed of sedimentation of a virus is a function of the size of its corpuscles. Ultracentrifugation therefore provides information about virus dimensions.*

(4) Electron microscope: *a microscope in which the rays of visible light used in an optical microscope are replaced by a stream of electrons. This allows very much greater magnification to be obtained. (See Vol. 1, pp. 30 and 31).*

(5) Electrophoresis: *measurement of the migration of electrically charged substances under the influence of an electric field. Substances carrying different electrical charges migrate at different speeds.*

specific receptors which are found on the surface of sensitive bacteria. A particular bacteriophage cannot lyse any bacterium; for example, one which is active against staphylococcus never lyses the cholera vibrio or typhus bacillus. An essential preliminary to lysis is attachment to bacterium. To bring this about, the bacteriophage must find an appropriate receptor at the surface of the bacterium, to which it always attaches itself by the extremity of its tail (fig. 4). Following this attachment, either nothing happens or all the phenomena pertaining to lysis take place. At the extremity of the tail is also located the mechanism which breaks down the bacterial wall. This digestion of the wall was revealed by Barrington and Kozloff, using radioactive isotopes; it prepares the way for the penetration into the bacterial cytoplasm of the deoxyribonucleic contained in the head of the corpuscle.

From this moment the protein envelope has accomplished its role. It remains an empty and useless sac on the outside of the bacterial wall (Hershey and Chase, 1952).

The small central mass of deoxyribonucleic acid from the head of the phage particle goes on alone to determine the process of multiplication of the bacteriophage and as a final consequence, the total lysis of the bacterial cell.

Bacteriophagic phases

Let us now examine more closely what happens when a bacteriophage corpuscle meets a sensitive bacterium (fig. 4). The chemical disulphide-amino groups ($S-S-HN_2$), which are in the distal portion of the tube of the tail, disappear on combining with the 'zinc' cations in the bacterial wall. In doing this they unmask the extremity of the axial cylinder which penetrates into the thickness of the bacterial wall. The proximal extremity of the sheath, which is composed of contractile protein, then retracts and thickens. It is at this moment that the limited digestion of the bacterial wall, of which we have already spoken, takes place. Then the small mass of deoxyribonucleic acid contained within the head of the corpuscle enters the body of the bacterium.

Four things can happen to the bacterium after injection of phage: the lytic cycle, lysogenization, transduction, or conversion.

The lytic or productive cycle

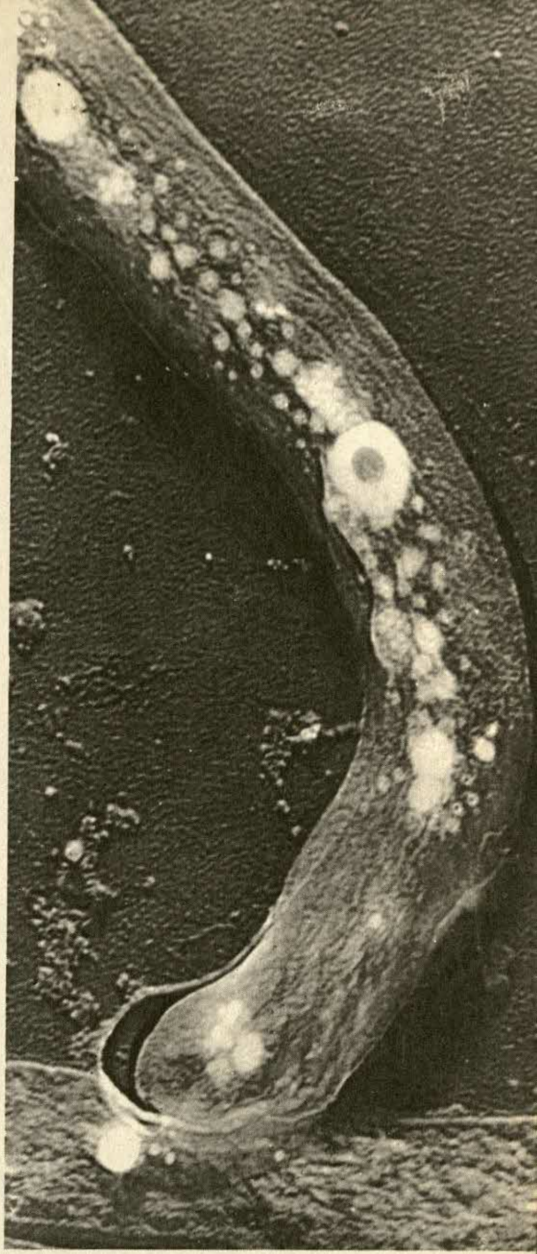
This is the infectious cycle observed in the case of 'virulent' phages.

Immediately after the penetration of the particle of deoxyribonucleic acid from the bacteriophage into the bacterium, the metabolism[13] of the latter changes completely and becomes orientated exclusively towards the production of the various substances which enter into the composition of the bacteriophage (figs. 1 and 2). First of all, there should appear everywhere in the bacterial cytoplasm numerous granules of deoxyribonucleic acid identical to that of the infecting bacteriophage. These granules become concentrated into a number of small masses around each of which protein molecules assemble and form the envelope and tail of new corpuscles. At this moment, the wall of the bacterium breaks and liberates these corpuscles into the surrounding medium (fig. 5). Thus the phage has multiplied or, to be more exact, everything seems to point to its having multiplied. In fact, we know that it is the infected bacterium that has made this so in a very large number of cases. The multiplication of bacteriophages is therefore very different from the reproduction of bacteria or protozoa. It gives an idea of how multiplication occurs in other ultra-viruses.

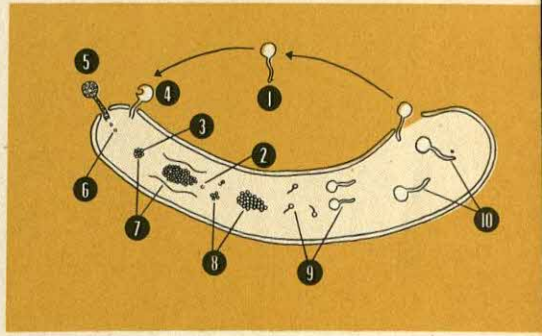
Reductive infection or lysogenization

Bacteriophages which are called 'temperate' can bring about another type of infection: the reductive infection or lysogenization in which some of the infected bacteria escape lysis and become 'lysogenic'. This means that they have acquired two new properties: an hereditary ability to resist lysis by the bacteriophage which has infected them, and a capacity, also hereditary, to make the same type of bacteriophage and liberate it in small quantities into the surrounding medium. These lysogenized bacteria are fairly similar to the spontaneously lysogenic bacteria which are found in numerous families of bacteria, particularly the enterobacteria.

Where does this strange property come from? Lwoff showed that if one cultivates a lysogenic bacterium (*Bacillus megatherium*) on a synthetic medium,[14] and then isolates it by micromanipulation[15] and washes it to get rid of free bacteriophage, this bacterium can no longer liberate bacteriophage. The lysogenic property is suspended but not totally abolished because as soon as the bacterium is replaced in a normal nutritive medium it produces bacteriophage as before. This shows that the lysogenic property is hereditarily perpetuated and that the presence of free bacteriophage is not essential for the transmission of this



The phage cycle in a bacterium: several stages shown in one bacterium: morula, rings of division, rosettes (mag. × 40,000). Below, a schematic representation of the complete cycle: (1) free phage; (2) merophage; (3) tropophage; (4) phage envelope; (5) adsorbed phage; (6) sporophage; (7) morula; (8) rosettes; (9) telophate; (10) mature phage.



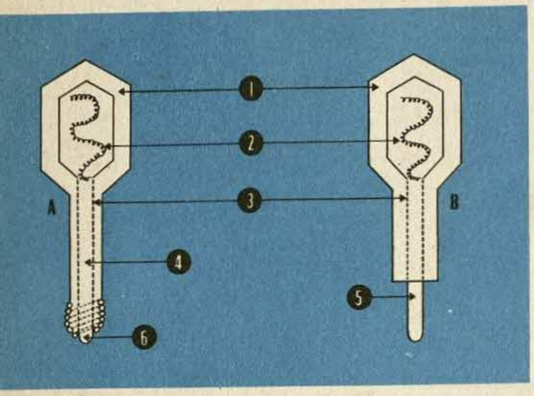


Fig. 3. (1) protein envelope of the head; (2) deoxyribonucleic acid; (3) protein of the tail, A. extended, B. contracted; (4) axial cylinder; (5) distol portion of the tube which disappears on the combination of the disulphide-amino groups with zinc. A. before fixation on a sensitive bacterium. B. after contact with its specific receptor present at the surface of a sensitive bacterium (after Evans, 1956).

characteristic. The property of making and liberating bacteriophage without any addition from outside can, depending on the circumstances, either come out into the open or remain suspended.

The prophage

The researches of Lederberg, Wollman, Lwoff and their team led to the idea that at the time of infection of a bacterium which is going to become lysogenic, the deoxyribonucleic acid of the infecting phage, instead of distributing itself in the cytoplasm and promoting the changes already described, becomes attached to the bacterial chromosome[16] at a specific point. From then on it becomes incorporated in the genetical apparatus of the bacterium. It is reproduced with it at each generation and is distributed into the daughter cells.

This fraction of deoxyribonucleic acid from the phage which becomes incorporated into the bacterial chromosomes was called 'prophage' by Lwoff. This prophage is neither infectious nor antigenic; it has the properties of a gene, since it is reproduced at each division of the bacterium.

It can be regarded as a potentially lethal gene which, in normal conditions and for most bacteria of the lysogenic population, does not become lethal. When it remains in the prophage state, that is to say, when the deoxyribonucleic acid of the phage remains in the bacterial chromosomes, not only is there no lysis but the bacterium is immunized against infection by phage.

How can the small quantities of bacteriophage constantly found in the medium of bacterial cultures be explained? Their presence is due to the fact that in a population of lysogenic bacteria mutations occur, usually at a very low rate, towards a return to the sensitive form. It is probable that in these mutants which have again become sensitive, the prophage has separated from the chromosome. It thus ceases to ensure the immunity of the bacterium and, again free in the cytoplasm, it determines the multiplication of the bacteriophage, the lysis of the bacterium and the extensive liberation of newly formed phage corpuscles.

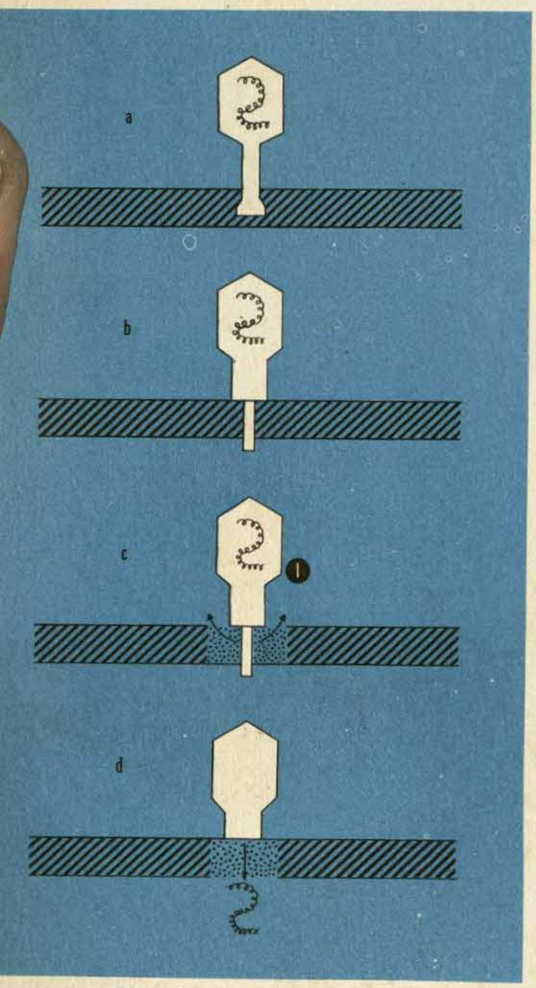


Fig. 4. (a) Fixation of a phage particle on the bacterial wall; (b) Modification which follows contact of tail extremity with bacterial wall; (c) Bacterial wall digestion limited to the point where the corpuscle is fixed; (1) contracted protein; (d) Passage of deoxyribonucleic acid from the head of the corpuscle into the cytoplasm. These stages constitute the adsorption of phage.

In certain cases the production of bacteriophage by the lysogenic bacteria can be the act of the whole or almost the whole of the elements of a bacterial population. If certain lysogenic cultures, called 'inducible', are submitted to the action of physical agents such as ultra-violet light or x-rays, or to chemicals such as thiomalic acid, ascorbic acid, hydrogen peroxide and other mutagenic and carcinogenetic compounds, like bis-methyl, B(chloroethylamine), all or almost all of the bacteria make numerous corpuscles. They undergo lysis and liberate a considerable quantity of free corpuscles. This phenomenon of the induction of lysogenicity was discovered by Lwoff, Siminovitch and Kjeldgaard in 1950.

Transduction and conversion

Sometimes the infection of a bacterium by a temperate bacteriophage is accompanied by the transfer to the former of a property coming from the bacterium which has served to regenerate the bacteriophage in question. If the recipient bacterium at the same time does not undergo lysis, this is a phenomenon called transduction.

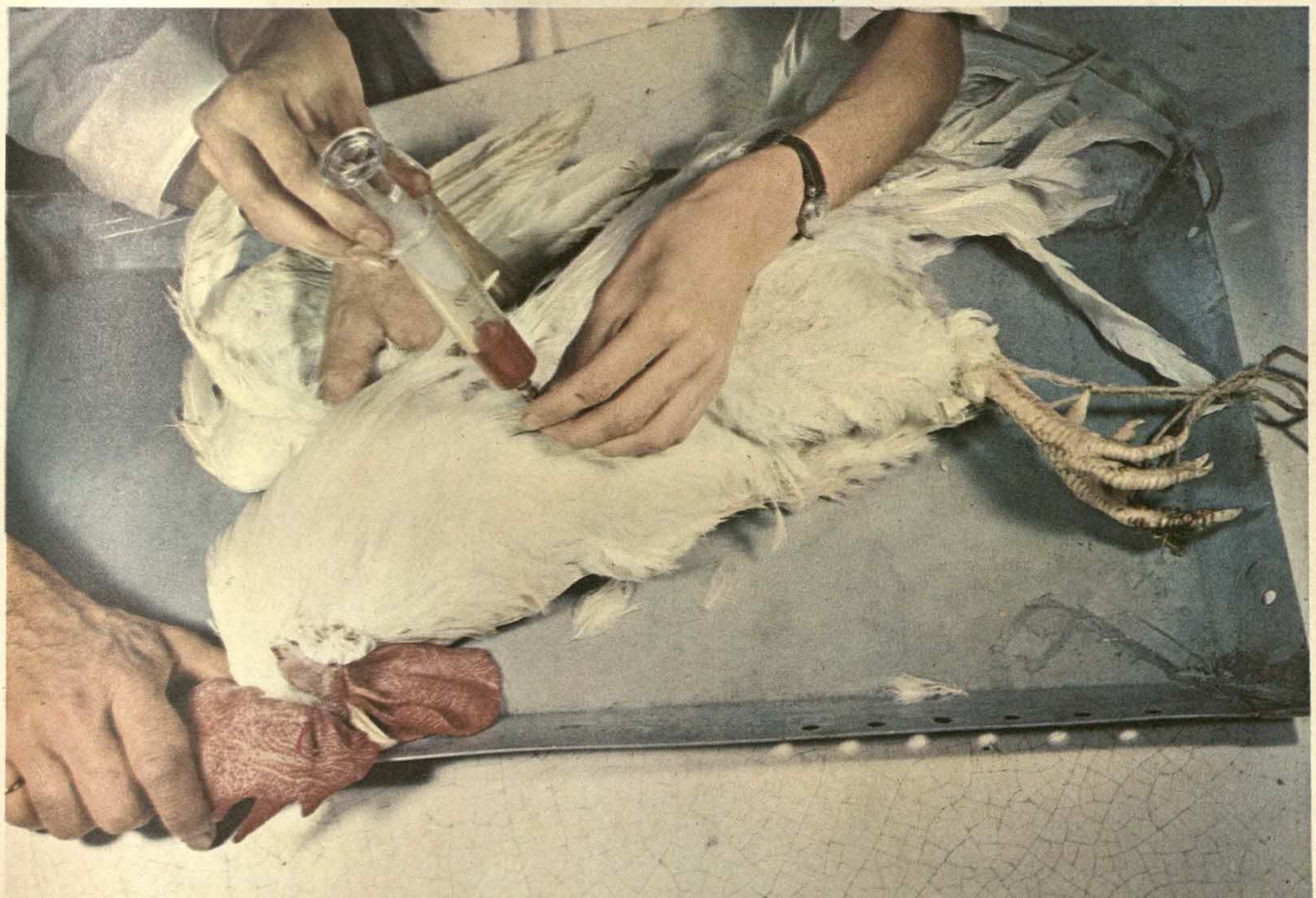
Conversion is the simultaneous acquisition by a bacterium of the property of being lysogenic and acquiring a new characteristic. In this case the two acquisitions are indissolubly connected and both are hereditary. Thus Freeman and his team of research workers in 1951 conferred the property of making toxin to diphtheria bacilli, which were non-toxin-forming and non-lysogenic, by treatment with a temperate bacteriophage extracted from a lysogenic and toxin-producing strain of the same species.

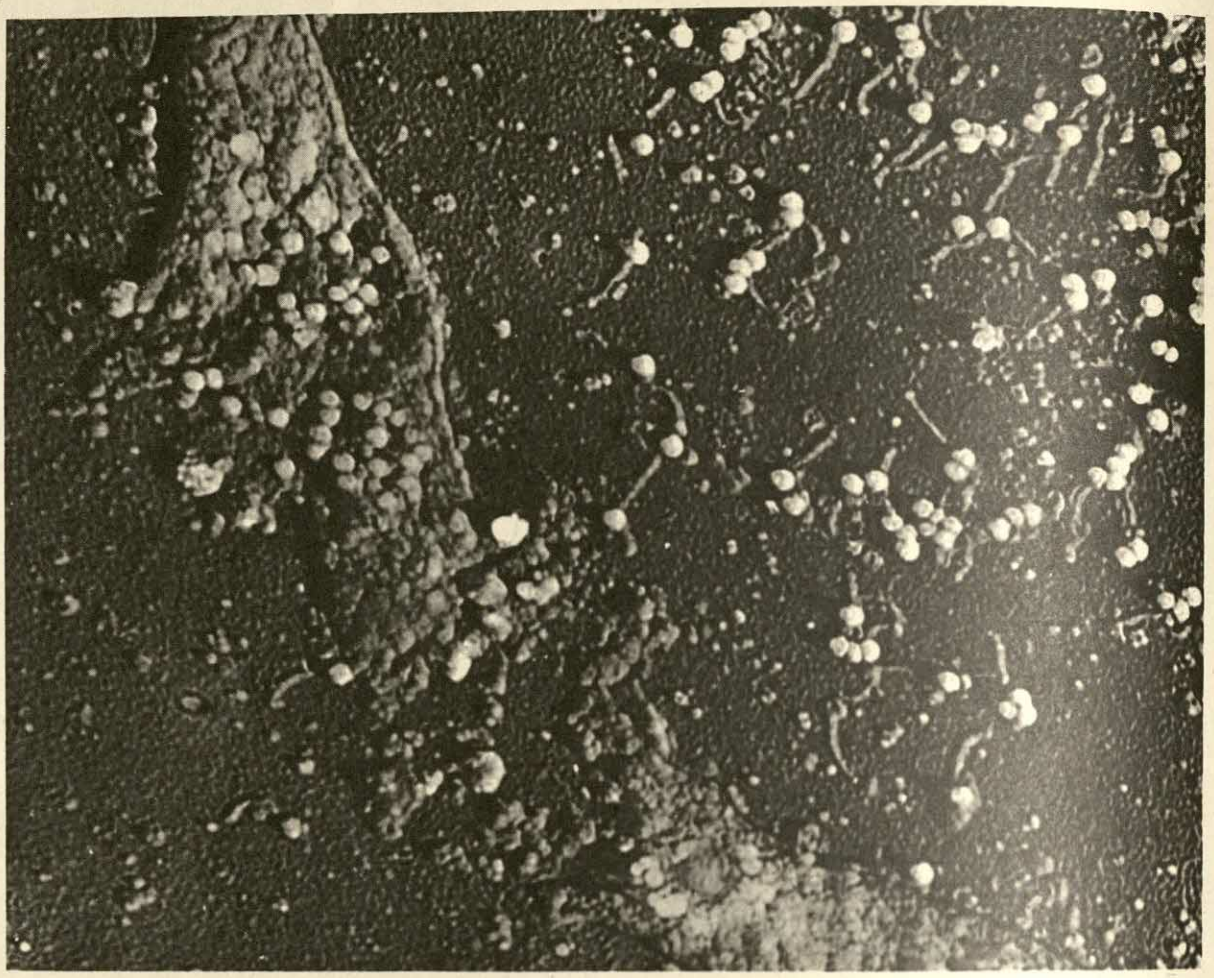
Similarly, the Japanese bacteriologists Iseki and Takai, in 1953, converted a nonlysogenic culture of *Salmonella anatum* (antigens '3' and '10'), by means of a temperate bacteriophage (phage E15) obtained from a lysogenic culture of *Salmonella newington* which possessed the somatic antigens '3' and '15'. The converted culture was identical with that of *Salmonella newington*, and possessed lysogenicity (phage E15) and antigens '3' and '15'.

Thus conversion differs from transduction inasmuch as the acquired character is always accompanied by lysogenization of the bacterium.

As we have seen, bacteriophages are viruses which destroy numerous species of bacteria. They are selective agents of bacterial

The problems of immunity have largely been solved by innumerable laboratory experiments in which various theories have been tested. Pasteur and Koch were the first to develop the basic bacteriological techniques. These pictures show (above) injection of a rabbit with a microbial culture; (below) the sterile removal of blood by intracardiac puncture in a chicken. (Photo Lod, at the Pasteur Institute at Garches.).





A bacterium infected with a bacteriophage proceeds to manufacture a large number of phage particles. When these reach maturity the bacterium bursts and liberates them; this is called lysis. Here a multitude of liberated phage particles can be seen, as well as residual matter from a bacterial cell after its destruction. (Mag. × 38,000. Istituto Superiore di Sanità, Rome).

mutants which have properties often very different from those belonging to the bacteria originally at work. By their powers of transduction and conversion, they are, in short, the agents of transformation of bacteria.

There is no reason to think that all these reactions which have been observed in test-tube experiments do not also take place in nature and within infected organisms (man, animals and plants) where sensitive bacteria and active bacteriophages are present together.

It is reasonable to suppose that bacteriophage play a very important role in the many unexplained processes of evolution in bacteria. So far as pathogenic bacteria are concerned, this role is in some instances favourable to the host, owing to the elimination of the pathogenic germs by lysis or as a consequence of selection in the direction of a lesser resistance by the germs to the host's usual means of defence.

In other instances, however, the bacteriophages, by their properties of transduction and conversion, may be responsible for the sudden appearance of virulence amongst bacteria which until then were harmless saprophytes.

Bacteriophagic action may also explain the heightening and decline of the epidemic characteristic of certain infectious diseases, in particular, plague (G. Girard, 1957).

In therapeutics, the bacteriophages are credited with success and notable improvements, but their action, as so often happens with biological medications, is rather inconstant.

The methods of lysotyping have considerably simplified the identification of certain bacterial 'types' among cultures belonging to a like bacterial species, and have rendered invaluable service to bacteriology and epidemiology, particularly as regards the typhoid and paratyphoid fevers, and staphylococci.

(6) Chromatography: *an analytical technique which allows the separation of a mixture of substances in solution because of their differential movement in a porous medium. To identify these separated substances 'developers' are used which give a characteristic colour.*

(7) Serology: *the study of the immunological properties of sera.*

(8) Immunochemistry: *the branch of chemistry dealing with conditions of immunity.*

(9) Radioactive isotopes: *certain elements (metals, metalloids) can be rendered artificially radioactive; they are then incorporated in more complex molecules. These can be injected into organisms or introduced into their food (into the media of bacterial cultures), and because they are easy to detect they are used to follow the fate of the substances which contain them.*

(10) Deoxyribonucleic acid: *a characteristic substance found in the nucleus of a cell.*

(11) Enzymes: *biological catalysts.*

(12) Antigenic properties: *certain substances called antigens, when injected into an animal, lead to the formation in the animal's serum of substances (antibodies) which have the property of combining with the antigen which has determined their formation, to give varying reactions.*

(13) Metabolism: *the totality of reactions which go on in a living organism, allowing it to live, grow, multiply and defend itself.*

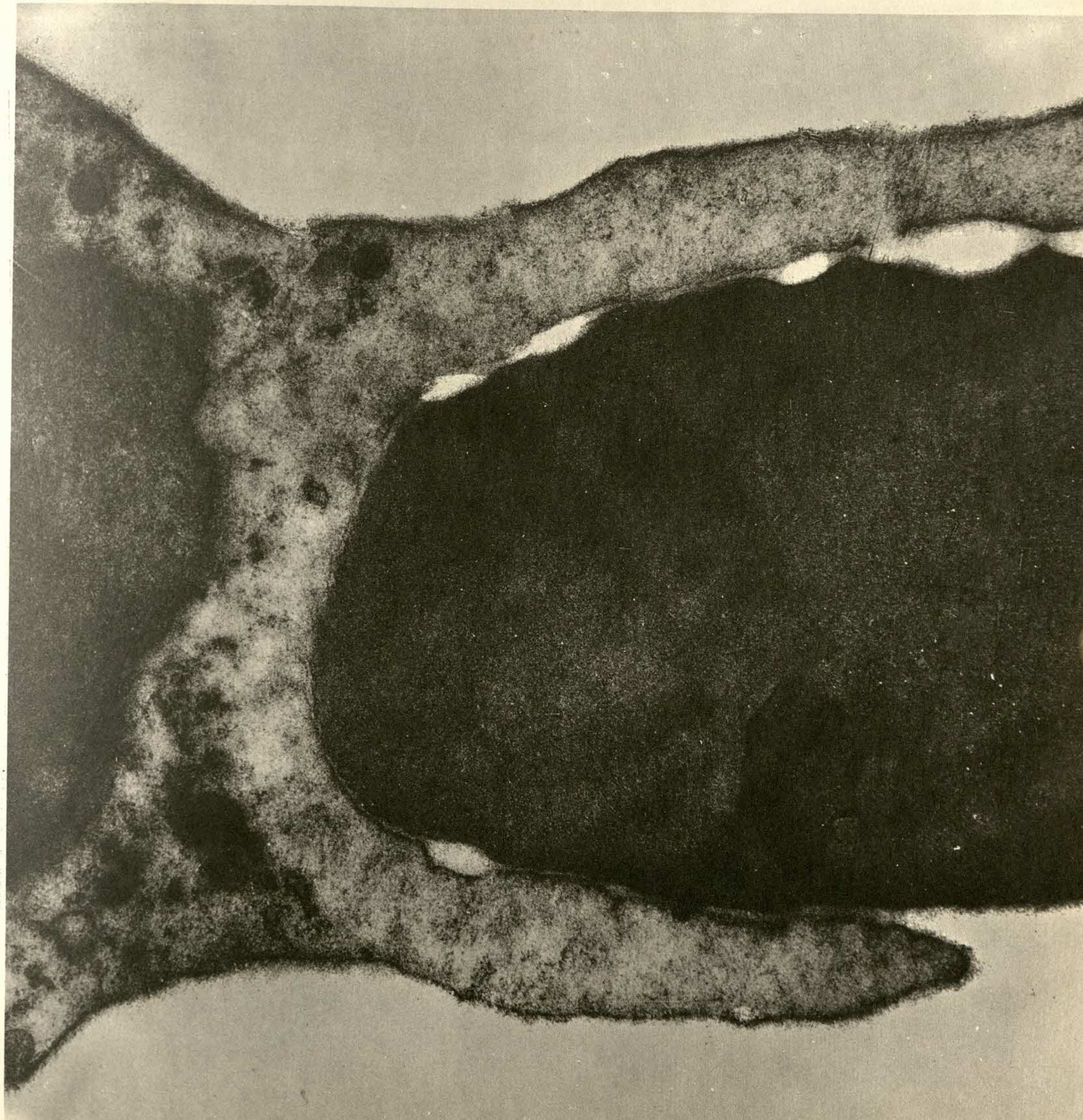
(14) Synthetic medium: *a culture medium which contains only chemically pure substances.*

(15) Micromanipulator: *an apparatus which allows delicate manipulations to be made under a microscope: eg, the removal of an isolated germ, dissection, removal of a part of a microbe, etc.*

(16) Chromosomes, genes, mutations: *see the chapter entitled 'Sexuality of bacteria'.*

PART FOUR

Natural and acquired immunity



A red globule (dark body)— phagocyte by macrophage. (Photo Bessis).

The phagocytes

Henry HARRIS

In all metazoa there are specialized cells (phagocytes) which are able to absorb and destroy by digestion extraneous particles which are abnormal intruders in the tissues. Bacteria are numbered among these particles. Their capture by the cells most often brings about their death.

For those who find it appropriate to explain biological phenomena in military terms, no subject is more attractive than the phagocytic[1] activity of cells in response to infection. In his original observations on the activity of these cells Metchnikoff was at once struck by the analogy between the responses of the tissues to infection and a field of battle, and his descriptions contain such terms as the 'invading' bacterium and the 'defences' of the host. These terms continue to be used in current text books and they are apt.

When bacteria are introduced into the tissues of an animal they provoke a series of events which eventually results in the arrival of large numbers of phagocytic cells at the site of the infection: this series of events is known as inflammation. The essential characteristic of inflammation, in whichever animal forms it occurs, is that it enables the phagocytic cells which circulate in the blood to emigrate from the blood vessels into the tissues at the site of the injury or infection. Once they are in the tissues, the phagocytic cells may destroy, by a process of ingestion and digestion, the micro-organisms which have been introduced, or they may fail to do so. Success on the part of the phagocytes results in recovery from the infection and repair of the tissues; failure results in spreading of the infection and the eventual death of the host.

The defence mechanisms

Phagocytosis is the normal feeding mechanism of a large number of protozoa,[2] and a layer of phagocytic cells forms the digestive tract of many primitive metazoa.[3] But even in the most primitive metazoa, such as the sponges, there is already a well-developed system of phagocytic cells whose function appears to be essentially similar to that of the phagocytes of higher organisms. A phagocytic system is thus a common feature of the defence mechanisms of virtually all animal forms, including those which do not have blood vessels. However, with the evolution of vascular[4] systems of increasing complexity, one sees a marked elaboration and specialization of the phagocytic system. Thus, in the primitive metazoa there is usually only one form of phagocytic cell, whereas in mammals, apart from the numerous specialized phagocytic cells found in the different organs, there are at least three types of phagocyte circulating in the blood. These blood-borne phagocytes are known as leucocytes.[5]

The various leucocytes are distinguished by differences in their shapes and affinities for certain stains. In man the commonest of the blood-borne phagocytes is the neutrophil polymorphonuclear leucocyte, so called because it has an irregular lobulated nucleus and its cytoplasm has no marked affinity for either acid or basic dyes; it is predominantly involved in the primary reaction to infection.

When the tissues are injured by infection with micro-organisms or by other means the capillaries near the injury dilate and the flow of blood through them is retarded. This retardation results in the adherence of the leucocytes to the inner wall or endothelium of the vessel. The leucocytes then move by amoeboid motion along the endothelium and eventually penetrate it, thus moving into the tissue. Recent electron microscope studies suggest that leucocytes emigrating from vessels may pass through the substance of the endothelial cells rather than between them.

As the leucocytes move through the tissue spaces they are attracted to the micro-organisms lodged there by substances produced by the micro-organisms themselves.

(1) Phagocyte: *a cell which absorbs particulate material by engulfing it within its cytoplasm.*

(2) Protozoa: *a group of animals that consist of one cell only.*

(3) Metazoa: *animals whose bodies are composed of many cells.*

(4) Vascular: *composed of, or belonging to, blood vessels.*

(5) Leucocyte: *one of the white cells of the blood.*

(6) Enzyme: *a substance which in minute amounts greatly accelerates the rate of a chemical reaction. Most chemical reactions in the body are mediated by enzymes.*

(7) Antibody: *a protein in the blood which combines specifically with foreign matter introduced into the body and renders it more susceptible to phagocytosis.*

(8) Autolysis: *dissolution of dead cells, due to the destructive action of enzymes which they contain.*

(9) Allergy: *abnormal hypersensitivity of the tissues to certain compounds.*

(10) Mucosa: *mucous membrane: a layer of cells containing some which secrete mucus.*

Such directed movement of cells, known as chemotaxis, has recently been studied in some detail in leucocytes. By the use of specialized optical devices the movements of leucocytes may be recorded on film as traces, and analysis of these traces permits one to determine whether a particular substance exerts a chemotactic effect or not.

A survey of micro-organisms shows that chemotactic substances are produced by a wide variety of different types, but some do not produce any chemotactic effect. The biological significance of the production or non-production of chemotactic substances is still obscure. It seems that some chemotactic substances are polysaccharides.

When the leucocyte comes into contact with the micro-organism, the latter may be taken into the cytoplasm of the cell, killed and digested. The precise mechanism by which particulate material is ingested by phagocytic cells is still obscure. In the case of large or elongated particles the cytoplasm of the cell can be seen to flow around the particle and engulf it, and a similar mechanism presumably operates for all particles. The particle thus enters the substance of the cell and subsequently undergoes solution.

There is now good evidence that, in at least some cases, the bacterium is killed within the cytoplasm of the phagocyte before it is digested. Some recent experiments suggest that specific bactericidal substances associated with the cytoplasmic granules of polymorphonuclear leucocytes are involved in killing the ingested micro-organism. Studies indicate that some enzymes,[6] which may be involved in the digestion of the micro-organism, are inactive against living micro-organisms but do act upon micro-organisms after they have been killed.

Bacterial defence and the formation of pus

The chain of events which has just been described may break down at any point. The leucocyte may be unable to ingest the bacterium, and this is often the case with encapsulated bacteria. There are some species of bacteria which have both encapsulated and non-encapsulated forms, the latter being readily ingested by leucocytes and the former not at all. Many bacteria which cannot ordinarily be ingested may be rendered susceptible to ingestion by the adsorption of certain substances on to their surfaces. The blood of animals may contain such substances, the most important of which are proteins known as antibodies.[7] These form the basis of acquired immunity to bacterial infections. The phagocyte may be able to ingest the micro-organism but unable to kill or digest it. The micro-organisms may produce substances which quickly kill the leucocyte. It will therefore be clear that the outcome of a bacterial infection will be determined by a large number of variables, and that the development of the lesion may take many forms.

If inflammation continues, increasing numbers of polymorphonuclear leucocytes will emigrate from the vessels and accumulate in the tissues. The phagocytes will be localized in the vicinity of the bacteria and many of them will be killed, so that a dense aggregate of living cells and dead cells will be produced, which may liquefy as a result of autolysis,[8] thus giving rise to pus.

The aggregation of leucocytes in and around bacterial colonies may also be demonstrated *in vitro.*

Monocytes and macrophages

While the polymorphonuclear leucocyte is the phagocyte predominantly involved in the response to acute infections, the monocyte, or macrophage, is the cell which predominates in chronic infections. There is good evidence that the monocyte and the macrophage are simply two physiological states of the same cell. When the cell is in the blood stream, it is referred to as a monocyte; but when it is found in the tissues its form is somewhat altered and it is usually referred to as a macrophage, a term coined by Metchnikoff to distinguish it from the polymorphonuclear leucocyte which he called a microphage.

In almost all animal species the number of monocytes in the blood is far smaller than that of polymorphonuclear leucocytes. The monocyte is considerably larger and has a round or crescentic nucleus in stained preparations. When the blood flow is observed in inflamed vessels, monocytes, as well as polymorphonuclear leucocytes, adhere to the endothelium. Emigration of monocytes through the endothelium has also been observed.

The mechanisms by which an accumulation of polymorphonuclear leucocytes is produced in the tissues have been described, but in chronic conditions such as tuberculosis and leprosy, accumulations of macrophages occur. One of the cardinal problems in the study of inflammation is how an accumulation of macrophages can occur when these cells are

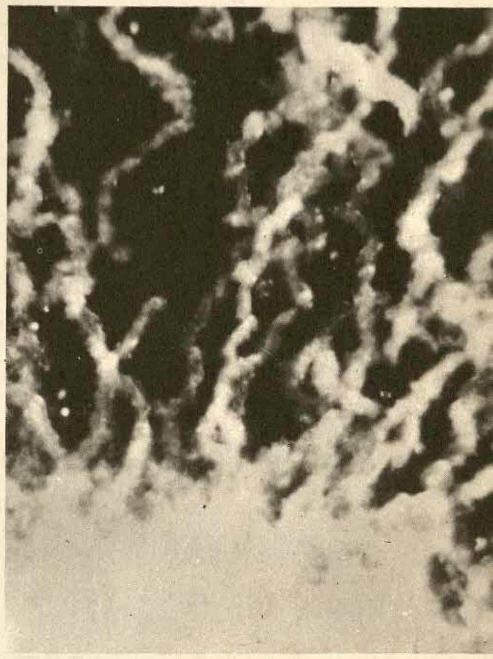
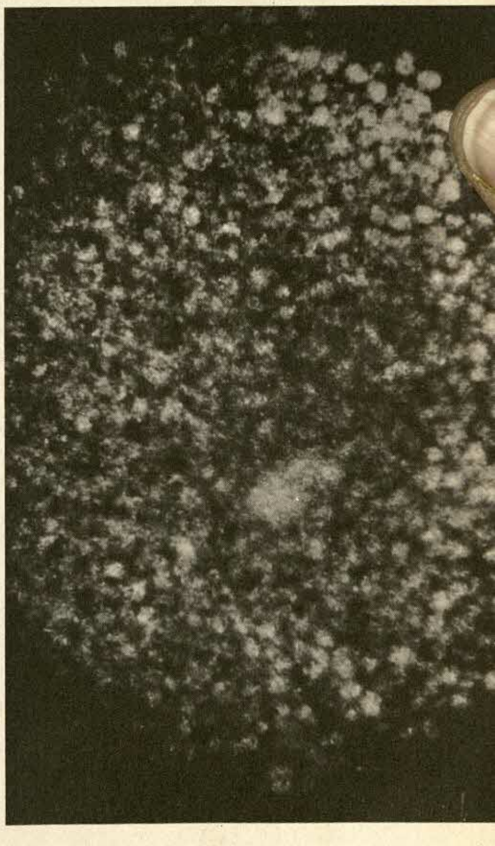


Fig. 1. In the presence of a mass of bacteria, all the tracks converge on the bacteria: the leucocytes show a positive, chemotactic reaction to the substances formed by the bacteria.

Fig. 2 (below). A bacterial colony almost entirely invaded by the polymorphonuclear leucocytes that have brought about the phagocytosis of its elements.



so greatly outnumbered in the blood by the polymorphonuclear leucocytes.

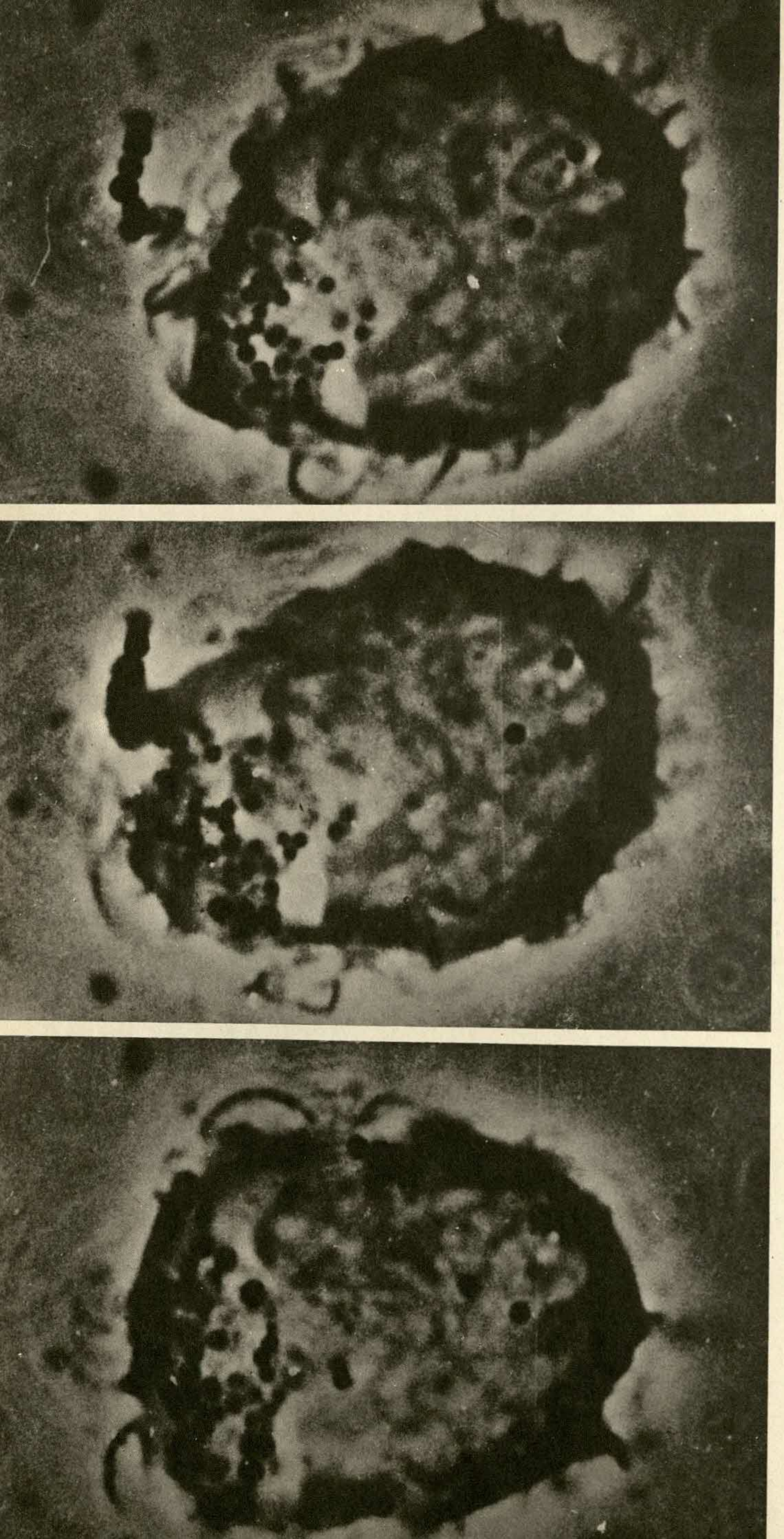
The chemotaxis of leucocytes

In the last few years the development of suitable optical methods and of techniques for keeping macrophages alive for long periods has permitted a re-examination of chemotaxis in these cells. The macrophages move much more slowly than polymorphonuclear leucocytes, but they exhibit chemotaxis unmistakably, for macrophages move at random in the absence of any chemotactic substance, whereas the tracks converge in a linear fashion on a bacterial colony. The chemotactic response is, if anything, even clearer than that of the polymorphonuclear leucocytes.

However, an examination of the kinds of bacteria which elicit a chemotactic response in macrophages did not yield the expected result, namely, that only those bacteria which were associated with accumulations of macrophages *in vivo* were chemotactic for macrophages. On the contrary, it was found that a wide variety of micro-organisms were chemotactic, including many which were associated *in vivo* with accumulations of polymorphonuclear leucocytes. In fact, no micro-organism was found to be chemotactic for the one phagocyte without also being chemotactic for the other. In short, chemotaxis was found to be a non-specific reaction to which both types of phagocyte were prone and it was elicited in both cells by the same types of compound. These experiments rendered it extremely unlikely that chemotaxis could be responsible for differences in the cellular responses in different forms of infection, and other explanations had to be sought.

The war of attrition

The two cardinal respects in which the macrophage differs from the polymorphonuclear leucocyte are that it has a much longer natural life span and that it is able to survive in a variety of adverse conditions to which the polymorphonuclear leucocyte succumbs. There is no evidence that the macrophage is able to ingest bacteria which the polymorphonuclear leucocyte cannot, or vice versa; extensive studies have shown that specificity does not rest in differences in phagocytosis any more than in differences in chemotaxis.



A classic example of phagocytosis: a group of staphylococci gradually attracted, then absorbed, by a living monocyte. (Mag. × 3,000. Photo Frédéric and Robineaux).

From the very beginning of the inflammatory response, when the first wave of polymorphonuclear leucocytes appears in the tissues, some monocytes are also to be seen, although they are greatly outnumbered by the polymorphonuclear leucocytes, as is to be expected from their relative rarity in the blood stream. In many infections the invading bacteria produce toxic substances which rapidly kill the polymorphonuclear leucocytes, either before or after the bacteria have been ingested. The macrophages are in general more resistant to these substances and therefore survive. The macrophages ingest not only the bacteria but also the dead polymorphonuclear leucocytes, so that their relative numbers in the tissues grow with increasing duration of the lesion. As long as irritation to the vessel walls continues, increasing numbers of polymorphonuclear leucocytes emigrate into the tissues, and despite a relative increase in the numbers of macrophages, pus continues to form. If irritation to the vessels ceases, further emigration of leucocytes does not occur and the initially polymorphonuclear lesion is gradually converted into one of macrophages.

The further development of the lesion depends on the relationships established between the invading bacteria and the macrophages. If the macrophages are able to destroy the bacteria, then the lesion is resolved and healing takes place; the macrophages remove any dead cells or debris. If the macrophages are unable to kill the bacteria, then a chronic lesion results in which the bacteria continue to multiply either within or outside the macrophages, or both. Chronic tuberculosis and leprosy are classic examples of infections in which intracellular multiplication of the bacteria continues within the macrophages.

It is clear from the sequence of events described above that there must be differences between the polymorphonuclear leucocyte and the macrophage in their ability to kill and digest bacteria which they have ingested. Curiously enough, there has been, until quite recently, very little systematic investigation of intracellular digestion in these cells. In the last few years, however, evidence has been produced that important differences in this respect do exist between the two cell types.

Only one other cell in the blood is known to be phagocytic, and this is the eosinophil leucocyte. This cell closely resembles the neutrophil polymorphonuclear leucocyte in having a polymorphic, lobulated nucleus, generally with two lobes; but it has in its cytoplasm large granules which stain readily with acid dyes.

The function of the eosinophil leucocyte remains obscure. It normally represents only a few per cent of the leucocytes present in the blood. When inflammation produces the emigration of neutrophil polymorphonuclear leucocytes, occasional eosinophil leucocytes emigrate also; and in bacterial infections the eosinophil leucocytes may be seen to be engaged in phagocytosis of the bacteria together with the neutrophil polymorphonuclear leucocytes. Studies of eosinophil leucocytes *in vitro* show that their rate of locomotion is slower than that of the neutrophil polymorphonuclear leucocytes, but more rapid than that of the macrophages. As far as chemotaxis and phagocytosis are concerned, the eosinophil behaves in exactly the same way as the neutrophil and the macrophage; it is attracted by the same kinds of compound as the other two cells and it ingests or fails to ingest the same range of bacteria.

It is possible that the primary function of the eosinophil may have little to do with phagocytosis, even though the cell is phagocytic. The eosinophil is characteristically seen in large numbers in tissue reactions associated with hyper-sensitivity or allergy,[9] for example, in the bronchial mucosa[10] in asthma, or in infestations with parasitic worms where tissue sensitization is a prominent factor. In these conditions large collections of eosinophils may be seen, but they do not appear to be functioning as phagocytes. What they are doing is not known.

There are in the blood of mammals two other leucocytes: the basophil leucocyte, which has an affinity for basic dyes, and the lymphocyte, which is also present in the lymph. It is not known whether the basophil is phagocytic; but the lymphocyte is not.

In general, the leucocytes of the blood play their phagocytic role only during inflammation, when they emigrate from the vessels. But almost all organs have populations of phagocytic cells which operate continuously under physiological conditions. These cells, which for the most part are specialized forms of macrophage, are collectively called the reticulo-endothelial system. They operate as a versatile system of scavengers which rids the body of unwanted material. In the spleen, for example, they dispose of broken-down red cells from the blood stream; in the lungs they remove inhaled particles. Very little is known about the factors which govern or modify their activity.

HISTORICAL NOTES

1843
ADDISON first described the passage of white blood cells through the walls of small blood vessels.

1869
COHNHEIM demonstrated that the formation of pus is due to the continued passage of white blood cells through the walls of blood vessels. The essential feature of acute inflammation was thus made clear.

1882
KOCH demonstrated that lesions of tuberculosis are characterized by the multiplication of bacilli within macrophages.

1889–92
METCHNIKOFF made clear that the white cells of the blood are phagocytes and elucidated the role of phagocytic cells in the defences of the organism against infection.

1891
LEBER showed that polymorphonuclear leucocytes show positive chemotaxis.

1891
EHRLICH distinguished the various leucocytes in the blood, by staining techniques.

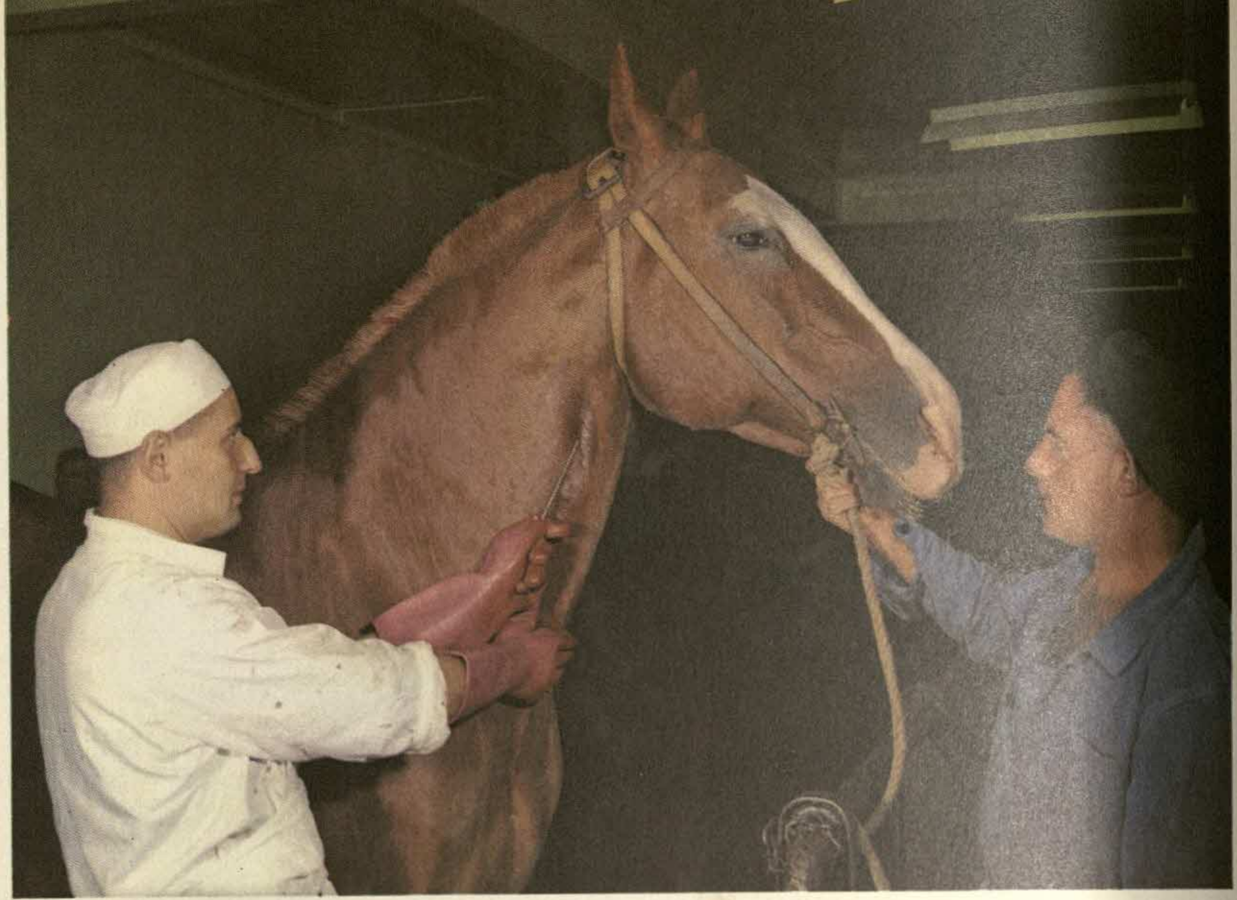
1903
WRIGHT and DOUGLAS showed that blood serum stimulates the phagocytic activity of leucocytes.

1934
MUDD, McCUTCHEON and LUCKE surveyed the phagocytic capacities of polymorphonuclear leucocytes and macrophages in vitro *and found that they are essentially similar.*

1953
HARRIS demonstrated chemotaxis in the macrophage for the first time, but showed (after DELAUNAY and LASFARGUES) that polymorphonuclear leucocytes and macrophages react chemotactically to the same substances.

1960
MACKANESS demonstrated unequivocal differences between the polymorphonuclear leucocyte and the macrophage in their ability to kill and digest microorganisms which they have ingested.

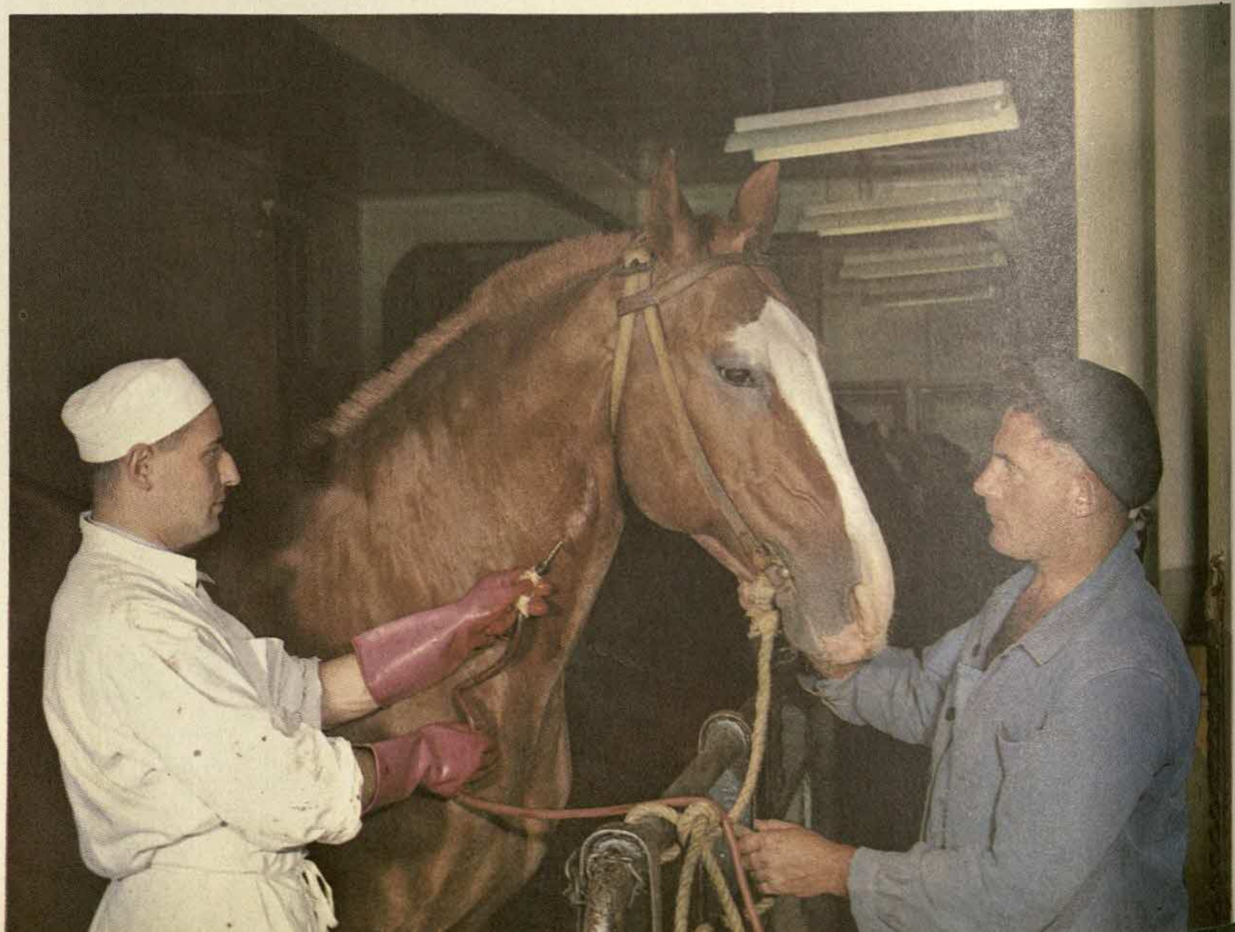
Serum is usually obtained from an immunized horse. This operation has been filmed at the Pasteur Institute at Garches, where the establishment comprises 500 horses. Here is the large needle which is used for the collection of blood.

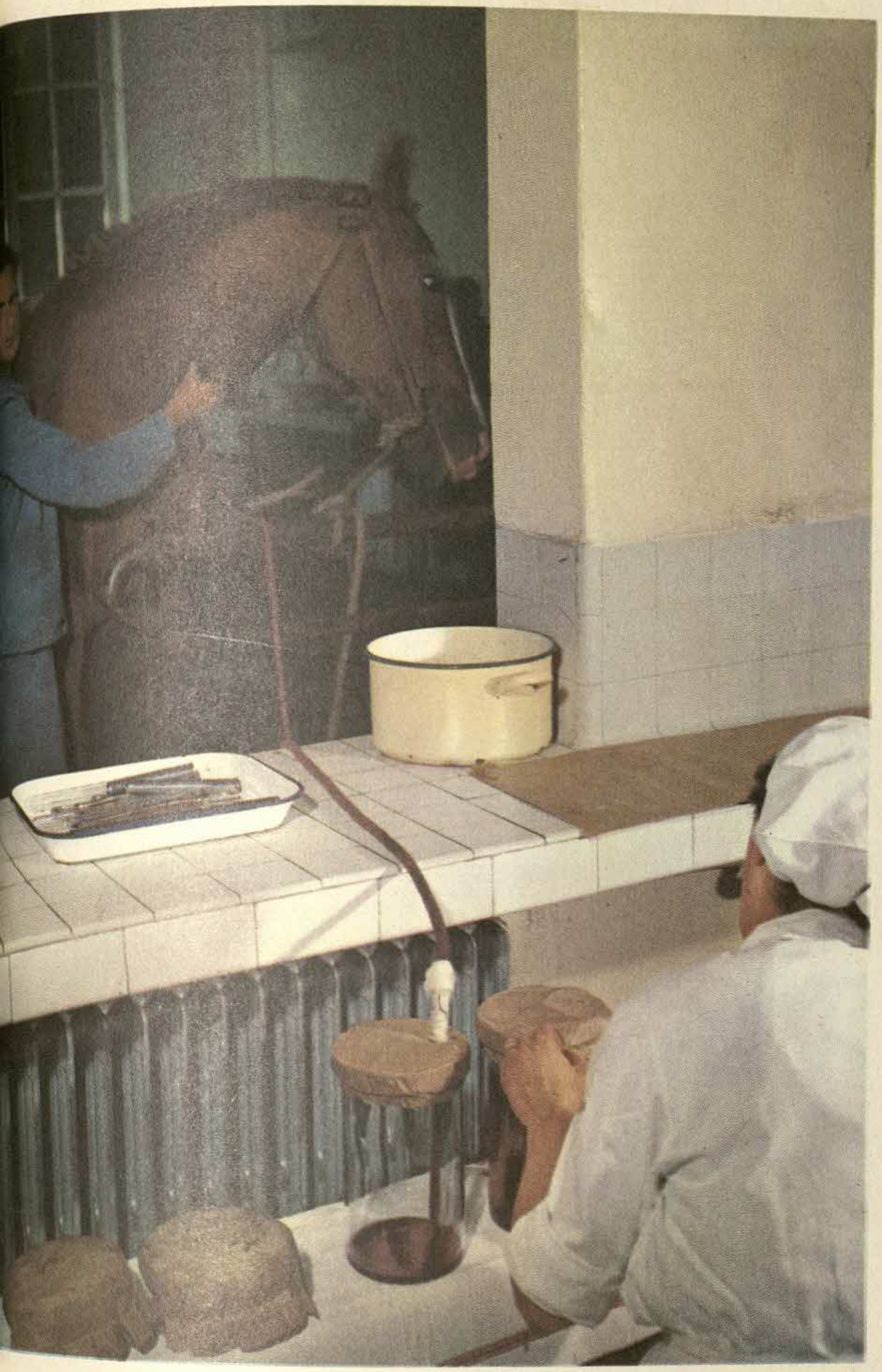


The incision is made in the jugular vein.

The collection of serum

The operator has employed standard aseptic technique.





The production of vaccines is an important contribution to the preventive aspect of the struggle against infections; that of sera is concerned with the curative aspect. The techniques of production have been gradually improved since the time of Pasteur, to whom we owe the first discoveries in this field. Today about thirty sera and vaccines are in current medical use. There are sera against diphtheria, tetanus, streptococci, meningococci, dysentery, colibacilli, venoms, anthrax, plague, pneumococci, gangrene, peritonitis and whooping cough. There are vaccines against diphtheria, tetanus, staphylococci, typhoid, paratyphoid, gonococci, pneumococci, cholera, meningococci, plague, yellow fever, bronchial complications of asthma, Rickettsia (agents of exanthematous typhus), whooping cough, tuberculosis (B.C.G.), acne and smallpox.

About seven litres of blood flow from the tapped jugular vein. The blood is collected, then placed in tanks at a constant temperature, eventually separating into two layers. The upper layer, the serum, is siphoned off. Some horses provide up to 400 litres of serum.

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The serum increases the reserves of the Pasteur Institute. It remains for six to nine months in a cold room which allows its strength to become stable. When needed, it will be distributed in ampoules. It forms a sort of blood bank, the security reserves of which should provide for the needs of the public. (Photos Lod).

Non-specific immunity

Giorgio CAVALLO

In order to oppose invasion by microbes the host organism, besides using phagocytes, antibodies and interferon, makes use of several chemical substances. One of these, properdin, has aroused great interest since its discovery in 1954. Another, lysozyme, the enzyme discovered by Fleming in 1922 and for a long time neglected, has again become the subject of very interesting work. This chapter deals with these substances and with another group, the bactericidins: these are ill-defined as yet, but the name that has been given to them indicates their properties.

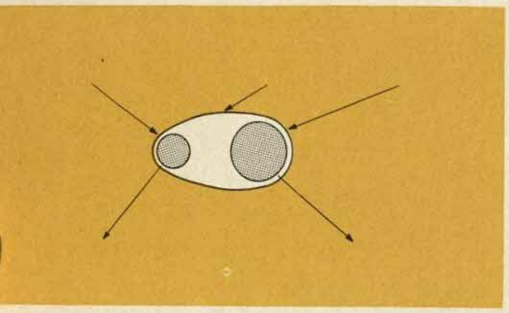


Fig. 1. Regulation of the level of properdin in the blood.

The use of lower fungi for therapeutic purposes is one of the striking advances of bacteriology. The circular, white-fringed colonies shown on the opposite page are of Penicillium (type Chrysogenum notatum) grown on medium p196. (Photo Lod).

Between 1880 and 1890, Nuttall, Buchner and Fodor observed for the first time the existence of factors that confer non-specific immunity. They showed in particular that human serum exercises a destructive action on bacteria—an action which decreases as the serum becomes older.

Nuttall also observed that the bactericidal property is considerably reduced by heating the serum at 56°, a treatment which does not affect the activity of antibodies that are orientated towards particular bacterial species and are the basis of specific immunity.

It was therefore concluded that the bactericidal property of serum depends on at least two different substances, one thermostable, specific and identifiable with the antibody, the other thermolabile and non-specific, which was given the name of 'complement' by Ehrlich.

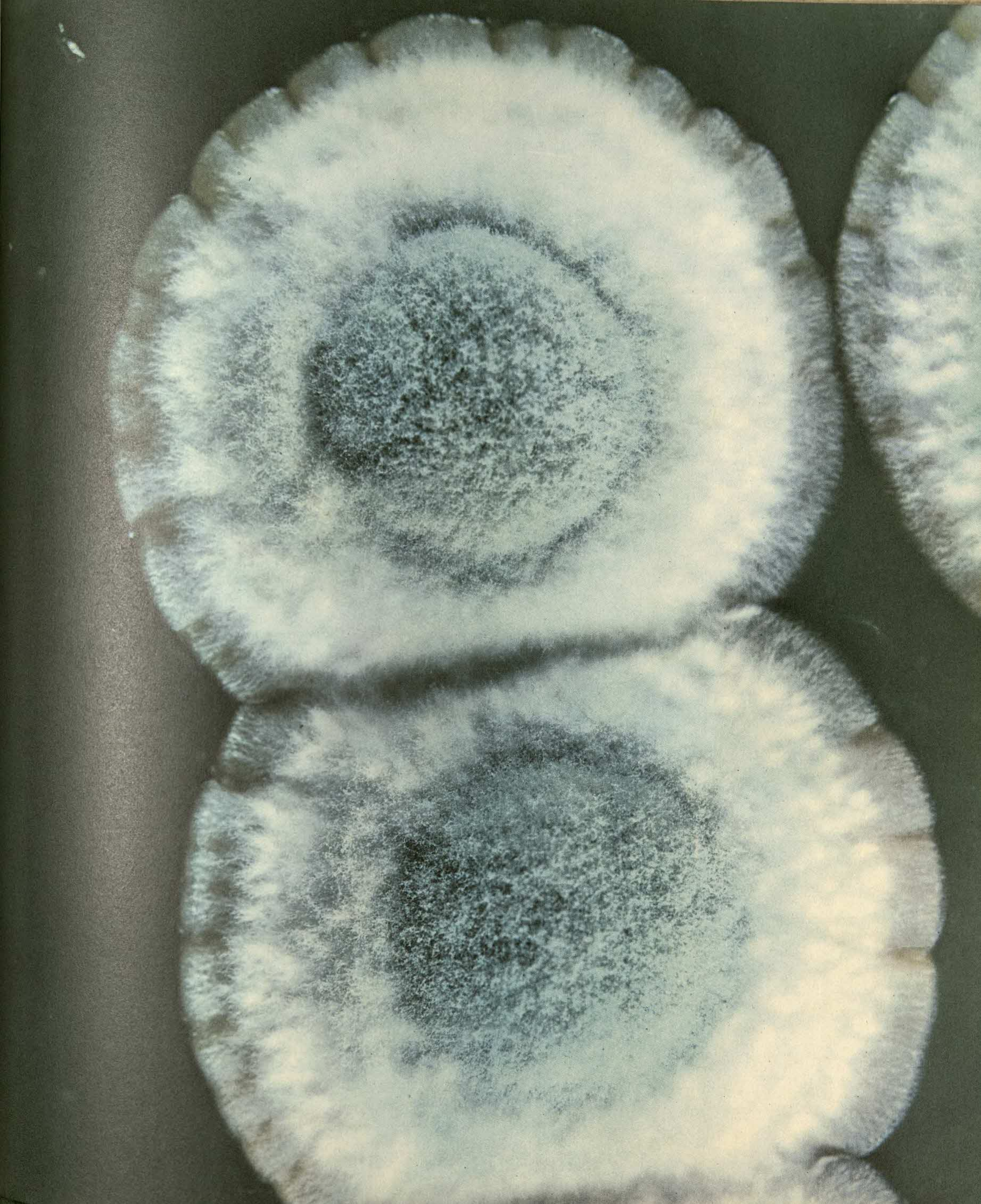
Further work showed the existence of several other non-specific immunity factors. Complement itself is not a single substance but is constituted by at least five different factors. These do not show any anti-bacterial activity on their own; in order for them to become anti-bacterial, additional substances are required which act in conjunction with the complement.

The non-specific humoral factors either act directly on the germs by killing them or indirectly by increasing the activity of the phagocytic cells.

These factors have been subdivided into three groups: the properdin system; the bactericidins; and lysozyme.

The properdin system

The properdin system owes its name to a protein which was isolated from human serum in 1954 by Pillemer and a team working with him. A large part of the bactericidal activity



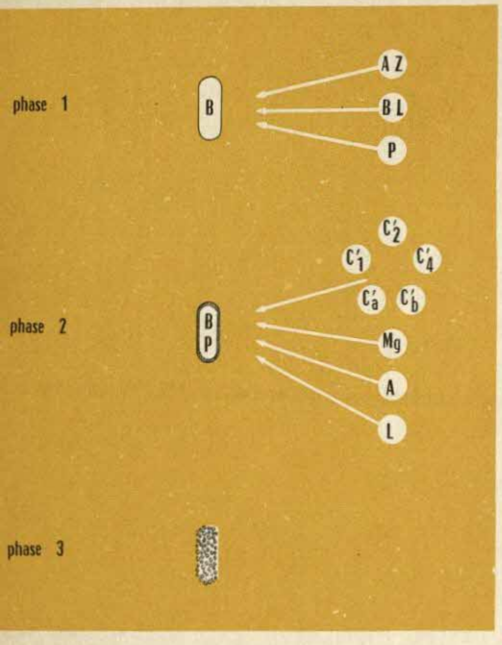


Fig. 2. Bacterial lysis by properdin takes place in three phases: fixation of the antibody, action of properdin, and the intervention of complement. The last stage is really responsible for the cellular lysis. B = bacterium, PB = properdinized bacterium, AZ = antizymosan antibody, BL = Blum factor, P = properdin, C_1, C_2, Ca, Cb, C_4 = the five components of complement, Mg = magnesium ions, A = factor A of Pensky, L = Leon factor.

of fresh serum is associated with this protein. The word properdin is of Latin origin: *pro*, in favour of, and *perdere*, to destroy.

Properdin, in fact, is only one component of a system which consists of at least nine factors, and on its own it does not possess any activity. On the other hand, when properdin is in the presence of other constituents of the system—among these the components of complement are the most important—it destroys several bacteria, including those which cause dysentery, typhoid and paratyphoid fevers, etc, and certain protozoa, including *Trichomonas vaginalis*. It also exercises a neutralizing action on certain types of filterable viruses.

Properdin is not only concerned with defence against infections; it also plays a part in the pathogenesis of certain infectious diseases, such as paroxysmal haemoglobinuria, a condition in which red cells are easily lysed by serum of normal or affected individuals.

Pillemer has shown that the properdin system is directly concerned with this type of haemolysis and although his view is not shared by everybody, it is strongly supported by the excellent therapeutic results obtained by using inhibitors of properdin, such as heparin, in haemoglobinuria patients.

The possible ability of properdin to inhibit the development of tumours in experimental animals is still much discussed. Two American research workers, Herbut and Kraemer, put forward in 1954 the hypothesis that properdin is one of the defence factors which higher organisms possess against tumours. These two experts, by experimentally reducing the blood level of properdin in rats with injections of zynosan (yeast extract), succeeded in making these animals susceptible to the graft of human tumours which normally have no hold on them. Other research workers, however, have more recently noted that all tumours do not behave in the same way and grafting does not invariably succeed after injections of zymosan.

Recent experiments have shown that a neoplasm causes a drop in the blood level of properdin during the course of its development. This phenomenon, in face of the results of Herbut and Kraemer, can be explained by considering the fall in properdin blood level as the effect and not the cause of the grafting of the tumour. This fall would depend on the reabsorption of polysaccharides released at the site of the lesion by the grafted cells, and also by the tissues damaged during the process of grafting.

The blood level of properdin is subjected to continual variations and its production and neutralization depend on numerous factors. Since 1954, it has been known that properdin is able to combine with zymosan, a polysaccharide extract of yeast, forming complexes. This phenomenon made the isolation of properdin possible. It is also known that several other polysaccharides of microbial or animal origin can combine with properdin. Among these are the cell-wall[1] polysaccharides of many bacteria, certain endotoxins, some dextro-rotatory and laevo-rotatory substances of bacterial origin, and inulin, as well as some animal kipopolysaccharides and mucins.

All these substances have in their turn been used for the isolation and purification of properdin. Certain of them, such as the bacterial lipopolysaccharides, are able to cause a considerable increase in the blood level of properdin. When larger quantities of these same lipopolysaccharides are given, however, a notable decrease of properdin may occur on account of the formation of large amounts of inactive complexes in the blood.

In these conditions, the blood level of properdin appears to depend on the presence of certain substances which may come from bacteria or food, or yet from the organs of the host itself, from which they are generally released in small amounts; but they may be liberated in considerable quantities in pathological conditions where there is tissue necrosis or after various types of shock.

The nature of properdin is still much discussed: according to Pillemer it is an enzyme: Nelson, on the other hand, considers it to be a particular type of antibody which is formed within the body in response to zymosan or similar substances.

In a paper which was published in 1960, Wedgwood, a pupil of Pillemer, arrived at an intermediate conclusion in suggesting that in the type of bacteriolysis considered here, either properdin, with its accessory factors, or a real antibody specifically formed against zymosan, is important. The destruction of bacteria is thought to take place in three successive stages: fixation of antibodies, the action of properdin, and the intervention of complement. This last stage would really be responsible for the cellular lysis (fig. 2).

Bactericidins

Myrvik has recently included in this group all the substances present in the serum of mammals which have a protective function

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(1) Cell wall: *one of the layers that encase the bacterial body; it is generally the thickest and most robust layer. The cell wall is composed of polysaccharides, lipids and proteins and is difficult to stain.*

against infection, the activity of which does not depend, even indirectly, on complement.

Certain bactericidins have been known since the last century; the first was discovered by Pane in 1891. The earliest investigations aroused hardly any interest and several years elapsed before further discoveries were made in this field. Eventually, a confusing situation arose because certain bactericidins had been discovered, so to speak, several times over, and on each occasion been given a different name. The factors included in this group generally attack Gram-positive bacteria, while those of the complement-properdin system act only on Gram-negative bacteria.[2]

Also included in the bactericidin group are the leukins, sometimes called endolysins, which are produced by the leucocytes. Some years ago it was thought that endolysins were able to act on Gram-negative bacteria as well, but Hirsch showed in 1956 that leucocytes produce another anti-bacterial substance, besides the leukins, which he called phagocytin. It would be this substance that is able to act on Gram-negative bacteria.

Substances which are similar to the leukins are the plakins, which are produced by the platelets.[3] And there are also the beta lysins, so called by Patterson in order to distinguish them from complement, which at the beginning of the century was called alpha lysin.

Lysozyme

In 1922, seven years before discovering penicillin, Fleming obtained a lytic principle from the nasal mucus of a patient with the common cold which was particularly active on certain non-pathogenic micro-organisms, including a bacterium which he isolated from the same patient and called *Micrococcus lysodeicticus.*

This lytic principle which, strictly speaking, should be classified amongst the bactericidins, has been identified as a protein of low molecular weight with enzymatic activity. It is present in almost all the body fluids, except urine, sweat and the cerebro-spinal fluid. It is now universally known by the name lysozyme.

If one allows a drop of this purified enzyme or of a fluid which naturally contains it (for example, a tear or some nasal mucus) to fall on a culture of *Micrococcus* seeded on to an agar plate, one sees that the culture develops over the entire surface except in the areas where the lysozyme was deposited. In these areas the bacteria undergo a lytic process; their multiplication ceases, the cells enlarge considerably, then their exterior layer, which the microbiologists call the cell wall, is destroyed, and within a few minutes only a few granules, hardly visible under the ordinary microscope, remain.

The lytic action of lysozyme on *Micrococcus* and other germs sensitive to this enzyme is clearly visible and extremely powerful. In fact, an infinitesimal amount added to suspensions containing a thousand million bacteria is sufficient to clear a cloudy cell suspension completely within a short time. This phenomenon does not apply to the majority of pathogenic bacteria, which are almost, or completely, insensitive to Fleming's enzyme.

For this reason many people have hesitated for a long time to attribute any importance to lysozyme as a factor in the mechanism of defence against infection: this opinion, however, is now being questioned.

Is lysozyme indispensable?

We do not know what would happen if man and higher animals did not possess lysozyme; one could speculate that certain bacteria which are sensitive to this enzyme are potentially pathogenic. That they do not succeed in invading higher organisms and producing disease may be because of their rapid destruction by lysozyme.

On the other hand, one cannot exclude the hypothesis that lysozyme, with the help of other immunity factors, acts on bacteria which are normally insensitive to it alone. This has been shown by Muschel to happen in the case of the typhus bacillus. This bacterium is completely lysed in a short time by the combined action of complement, specific antibodies and lysozyme, but none of these three factors when tested individually succeed in damaging the bacterial structure. There are also germs normally insensitive to lysozyme, such as *Escherichia coli*, which, under particular experimental conditions, can be completely destroyed by it. The favourable influence of chelating agents must be mentioned in this connection.

It is true that these observations can be made only *in vitro*, but there is no reason to believe that similar phenomena cannot take place in the organism of man and higher animals, since the number of substances that make bacteria sensitive to lysozyme is certainly greater than is at present known. All this now leads us to consider lysozyme as an element of some importance in the natural defence mechanism.

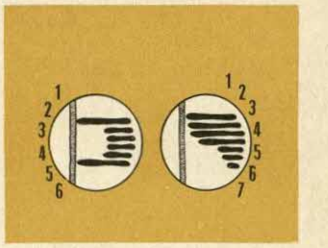

Fig. 3. Inhibition of different bacterial species by lysozyme, compared with inhibition by penicillin. The black bands represent the bacteria; their length is proportional to their resistance to lysozyme or penicillin, contained in the two vertical channels. Penicillin: (1) E. Coli*; (2) staphylococcus; (3) gonococcus; (4) streptococcus; (5) diphtheria; (6) typhus. Lysozyme: (1)* E. Coli*; (2) typhus; (3) staphylococcus; (4) megatherium; (5) sarcina; (6)* Bacillus subtilis*; (7)* Micrococcus lysodeicticus.

(2) Gram-positive and Gram-negative bacteria: *bacteria are divided into two groups. If, when stained with gentian violet and iodine-potassium solution, they resist decolorization by alcohol, they are said to be Gram-positive. Bacteria which do not resist decolorization by alcohol are said to be Gram-negative.*

(3) Platelets: *the smallest formed structures in the blood. They are spheroidal and are sometimes called thrombocytes because of their predominant role in the process of coagulation.*

Resistance to infections

Romolo DEOTTO

Within the same species, some individuals can be sensitive to the attack of a given micro-organism, whilst others are spontaneously proof against it, irrespective of previous immunization. The explanation of this phenomenon is complex, depending as it does on factors such as heredity, hormones, vitamins, and so on. It is also necessary to take into account the influence of environment.

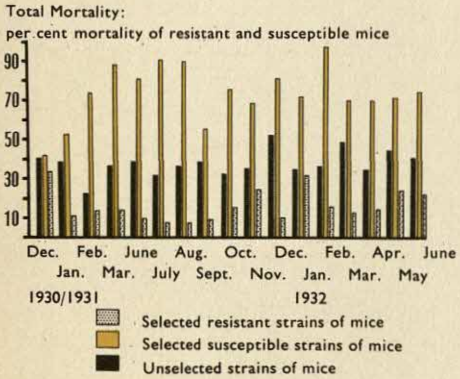

Fig. 1. Variations in the mortality of selected and unselected mice of a Rockefeller Institute Strain, after oral administration of S. enteritidis. *(L. T. Webster, J. exp. Med., 1933).*

It can be said that an infectious disease is, in fact, an instance of parasitism: a higher organism is attacked by a micro-organism, bacterium, virus or protozoan. But for a disease to establish itself, not only must the invading organism be pathogenic, the host must be receptive; one animal species may be resistant to a micro-organism which is pathogenic for another species.

However, even the receptive species opposes the infecting agent with a natural resistance; that is to say, a resistance which depends on the normal physiological condition of the individual and is not related to that which develops after contact with the infecting agent (acquired resistance or immunity).

This primary resistance comprises numerous defence mechanisms. Some, such as phagocytosis[1] and the bactericidal power of different humoral factors, are already known, but others are still unknown. Quite apart from pathological processes the degree of resistance varies not only according to race and individual characteristics, but also according to age, environmental conditions and diet.

Just as one can, in certain cases, relate the clinical varieties of a disease to varying properties of the infecting germ, so it is also possible to relate them to numerous forms of natural resistance which different individuals possess. It can be said that two genotypes[2] determine the clinical form of an infectious disease, that of the infecting bacterium and that of the infected organism.

The study of hereditary factors

Clinical and epidemiological experience has long shown that some races and groups of families are more readily disposed to certain infectious diseases than others. This observation does not necessarily mean that we can automatically attribute this diversity of natural resistance to hereditary factors. When speaking of the characteristics of a race, we cannot neglect geographical, social, nutritional and hygienic factors. All these factors are difficult to dissociate from the genotypes themselves and may increase, diminish or even turn aside reactivity. One must always be aware of the danger of attributing to hereditary factors reactions which are determined only by environment. However, careful experimental research and detailed statistical study of clinical and epidemiological data should

(1) Phagocytosis: *the property of certain cells present in different organs, and of certain white blood-cells, to ingest various matter such as particles of dust and micro-organisms. Among these cells are the microphages and the macrophages, so called according to their size.*

furnish reasonably precise answers to such questions.

When one wishes to examine the natural resistance which families of animals, raised from different strains, offer to a given infecting agent, one must first know whether the animals have ever previously been in contact with the pathogenic agent in question; otherwise, acquired immunity would confuse the study of the natural defence mechanisms. It is for this reason that Webster, in his experiments with mice, took great care to raise strains which had never been exposed to the infections that he was studying, and which therefore did not possess acquired immunity. Among these strains some appeared to be sensitive and others less sensitive to experimental infections by *B. aertrycke* (rats typhoid) and *B. enteritidis*. Moreover, the progeny of the mice which survived infection by *B. aertrycke* showed evidence of increased resistance to this infection as compared with the progeny of unselected mice. On the other hand, the offspring of the mice which had died from the infection showed increased susceptibility.

Starting from a hybrid strain of albino mice raised in the Rockefeller Institute—a strain which under standard conditions showed an average 37·4 per cent incidence of mortality after natural infection by *B. enteritidis*—Webster made numerous crossings. He interbred within two different groups, on the one hand using the offspring of mice which had survived and, on the other, the offspring of mice which had succumbed. By doing this he evolved strains of mice with a mortality incidence of 15 per cent and 85 per cent respectively. By a similar process of selection, but starting from different original strains, Webster obtained strains of mice with mortality levels of 1·89 per cent and 97 per cent respectively. Further cross-breeding between the very resistant and the very receptive strains showed that the factors for resistance and receptivity were transmitted in accordance with the laws of heredity.

Gowen and his research team in the laboratory of genetics at Iowa State College carried out further investigations in this field. They studied the natural resistance of rats infected by *Salmonella typhi murium* and of chickens infected by *Salmonella gallinarum*, and succeeded in selecting six strains of rats and five strains of chickens which showed different degrees of natural resistance to the corresponding *Salmonella*. After infection by 200,000 bacterial cells, the percentage survival was of the order of 8 per cent for the most susceptible strains of rats and of 13 per cent, 58 per cent, 75 per cent and 86 per cent for the other strains. Similarly, in the chickens survival was of the order of 1 per cent, 66 per cent, 76 per cent, 82 per cent and 90 per cent for the five strains respectively. These different resistances remained unaltered during successive generations, provided that the rats and chickens did not come into contact with the infecting agent. It was shown that the differences in reactivity were closely linked with the genotype of various strains of rats and chickens, the natural resistance to infections being determined by a combination of characters, each obeying its own hereditary law.

To state that natural resistance is linked with hereditary factors does not imply, however, that we are able to identify the individual characters which determine this resistance. Genetic factors influence cellular mechanisms which are not always easily revealed.

In the case of Gowen's rats, for example, one does not find factors capable of furnishing the explanation of resistance considered as an hereditary character. The only correlation that has been observed is between the degree of resistance and the number of leucocytes[3] present in the blood of different strains of rats.

Strains of rats with high resistance react to the infection by a leucocytosis[3] whose effectiveness surpasses that seen in more receptive strains (fig. 2). This is true for the macrophages[4] of the liver and spleen, which in the resistant strains produce enzymes capable of rapidly digesting ingested bacteria; whereas in the less resistant strains bacteria are not digested but increase.

Similarly, in chickens, the leucocytes of the more resistant strains phagocytize and digest bacteria rapidly, but in more receptive chickens the leucocytes digest bacteria more slowly.

These studies show that natural resistance is the effect of specific genes which act by controlling the production of bacteriolytic enzymes in the cytoplasm of phagocytes. This is one of the mechanisms of natural resistance —if not the only one—which shows the role of hereditary factors.

Bang and Warwick have used an entirely different technique and material for study, to show the effect of the hepatitis virus of the rat on macrophages obtained from the liver or other tissues of new-born rats, and cultivated *in vitro*. The macrophages from tissues of sensitive strains of rats are destroyed by the virus, whereas macrophages from strains resistant to the infection are not.

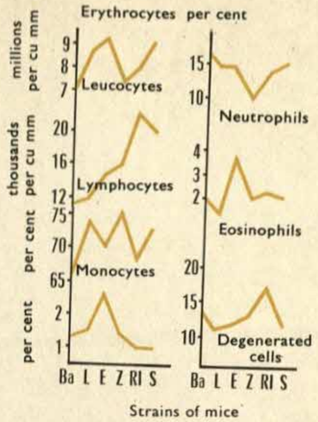

Fig. 2. Relation between resistance to typhus and the number of varying blood cells in different strains of rats. Abscissae: strains in order of increasing resistance. Ordinates: percentage numbers of the different cells. Note the parallelism between the increase in leucocytes and the increase in survival. (Gowen and Calhoun).

(2) Genotype: *a group of individual characters strictly linked to hereditary genes.*

(3) Leucocytes: *white blood cells.* Leucocytosis: *an increase in the number of white blood cells, for physiological or pathological reasons.*

(4) Macrophages: *see note 1.*

(5) Sickle cells: *cells which have the form of a sickle; red blood cells assume this form in sickle-cell anaemia.*

(6) Haemoglobin: *respiratory pigment contained in the red blood cells; during the pulmonary circulation haemoglobin fixes atmospheric oxygen, which it then carries to the tissues.*

(7) Homozygous: *a subject is said to be homozygous for one or more characters when it has received the gene for the character from both its parents.*

(8) Monochorionic: *twins coming from one fertilized egg; that is, from the same egg and the same spermatozoon. They have an identical genetic patrimony.* Dichorionic: *twins arising from two separately fertilized eggs.*

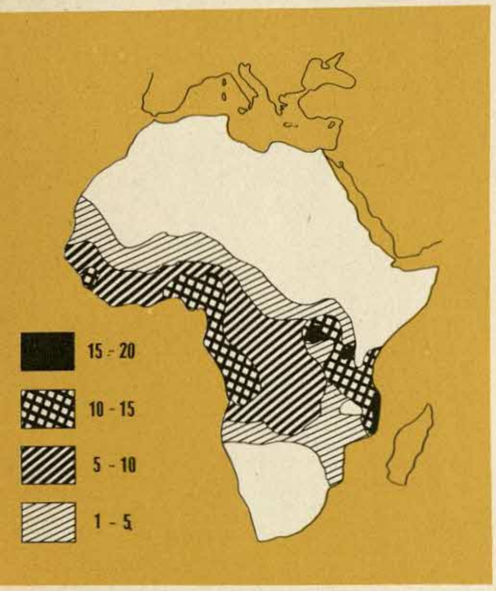

Fig. 3. Frequency of the sickle-cell gene as a percentage of the total population. The areas of greater frequency correspond to intensive tertiary malignant fever. (Allison, The Scientific American*).*

(9) Paratype: *a group of characters belonging to the environment and exercising an influence on the appearance of hereditary characters.* Phenotype: *the integral individual type is the resultant of the genotype and the paratype.*

Natural resistance in man

The study of hereditary factors which determine natural resistance in man is infinitely more complex than the study of such factors in animals. Nevertheless, this study has yielded some important contributions to the subject, although some of the results seem at first to be paradoxical. For example, it has been observed that a genetic situation, abnormal in that it can induce a morbid manifestation, can at the same time confer increased natural resistance against particular infecting agents.

Such is the case with sickle-cell anaemia,[5] a disease which is certainly hereditary and is characterized by the synthesis of an abnormal haemoglobin[6] (haemoglobin S). The red blood cells which contain this haemoglobin are, however, more resistant to the plasmodium of malaria than are normal red blood cells. Allison's observations of this phenomenon are as conclusive as data obtained in a well-executed and successful experiment with animals.

In a normal environment, without malaria, the number of people with sickle-cell anaemia is very small, and this number tends to decrease, because the homozygous[7] subjects do not generally reach maturity, owing to the gene which carries the pathological character; in areas where malaria is common, these same individuals have the advantage over normal subjects in face of the infection, and represent a relatively high percentage of the population. The areas where sickle-cell anaemia is more common correspond to malarial regions or regions which were malarial in the past (fig. 3).

It is the same in the case of Mediterranean anaemia or thalassaemia, another hereditary disease characterized by the formation of an abnormal haemoglobin. Ceppellini showed that thalassaemia in Sardinia is more common on the plains, where until only a few years ago malaria had raged for centuries, than in the mountains, where it is relatively rare.

Tuberculosis and other diseases

Tuberculosis is a widespread disease, the epidemiology and clinical development of which depend to a large extent on environment and nutrition. It has been clearly shown that in the case of tuberculosis there exists a natural resistance which is determined by hereditary factors. It is known that certain races, the Jews, for instance, are only very slightly predisposed to tuberculosis; this phenomenon is probably an expression of selection which has operated through the centuries.

A clear proof of the part played by hereditary factors in natural resistance to tuberculosis has been provided by Diehl and De Verschuer. It is a known fact that children of tuberculous patients are often subject to tuberculosis. In order to find out whether this was due to contagion, or because the children of tuberculous parents had a hereditary predisposition to the disease, Diehl and De Verschuer studied the behaviour of monochorionic (identical)[8] and dichorionic (dissimilar) twins. They found that, in the former, the disease appeared in nine cases out of ten in each twin; on the other hand, in dissimilar twins, only one in forty-two cases showed incidence of the disease in both of them.

It has also been shown that natural resistance to paralytic poliomyelitis and to petechial typhus is dependent on hereditary factors.

If we reflect on the variety and complexity of the mechanisms which allow an infecting agent to produce a pathological condition, we can easily visualize the number and variety of factors which constitute the natural resistance to a particular agent, alongside the mechanisms of cellular and humoral defence already mentioned. Even acquired immunity, which we have not considered here, shows clear associations with hereditary factors.

Influences of constitution, age and sex

Whether by individual constitution one means simply the genotypic component or, following the French and Italian schools, the collection of characters determined by both genotype and paratype[9] (identifying, in this case, the constitution with the phenotype), it is clear that natural resistance is influenced by the individual's constitution, since this is first of all the expression of hereditary factors. Thus, the asthenic constitution which is a variation of the leptosomatic constitution is, because of its weakness, particularly receptive to tuberculous infection, and in its most typical form might well be called a phthisic constitution.

The constitution may itself be pathological and show a reduced specific or non-specific resistance to infections; lymphatism, for example, is associated with a reduced resistance to tuberculosis, diphtheria and scarlet fever.

There is also a natural disposition of particular organs to infection; the apex of the lung, which is less mobile and also less aerated, lends itself easily to the localization of the tubercle bacillus; the appendix, which is fairly immobile and largely excluded from the free circulation of the intestinal contents, lends itself easily to infectious localizations; the bladder of the female, more than that of the male, is exposed from infancy to infections because of the smallness of the female urethra. The individual's constitution can strengthen or diminish these natural dispositions.

The relationship between age and resistance varies according to the infection. The high incidence of inflammation of the middle ear in children is due to the easier communication in the young between the bucco-pharyngeal cavity and the middle ear, which facilitates the penetration of micro-organisms.

The fact that children are more susceptible than are adults to gastro-intestinal infections, tuberculosis, whooping cough, measles, scarlet fever, roseola, can possibly be explained by an acquired immunity to infection which becomes slowly established over the years and makes the adult more resistant (fig. 4). But one cannot exclude a genetic mechanism for the development of a resistant condition.

Very often, we observe a change in the clinical picture of a disease rather than an increased resistance to infection, as in tuberculosis in children, adolescents and adults respectively; the same may be said of the Rous virus, which in new-born chicks produces a degenerative haemorrhagic disease, but which in adult birds produces a typical malignant tumour. Natural resistance, although hereditary, is not necessarily congenital; it can progress with the development of the embryo and with age through a natural process of maturation and differentiation of the tissues, or under the influence of hormonal and nutritional factors. For example, the pigeon at hatching is sensitive to anthrax but later becomes resistant to it. Again, it has been observed that embryonic tissues of the chicken's egg are easily infected by many viruses, though the adult birds and even new-born chicks are not affected. Similarly, exanthematous typhus is less acute in children than in adults.

Differences which are clearly related to sex have been noted in the response to diseases; but it is not yet possible to say whether these differences can be attributed to differences in natural resistance resulting from sex-linked characters, or whether they are simply a consequence of the different ways of life of the two sexes (occupation, working environment, hygienic habits, exposure to contagion, intoxication by alcohol, tobacco, etc).

It is nevertheless certain that male chicks are more resistant than female ones to infection by *Plasmodium gallinaceum*, the malarial agent of chickens; it seems that we cannot attribute this to the difference of equipment in sex hormones.

Hormonal equipment is yet another expression of the individual's constitution; it exercises a certain influence on the degree of resistance to infection, though it is difficult to tell the particular importance to be attached to each hormone[10] in the light of the numerous and reciprocal hormonal influences and their many and complex effects on metabolism.

The role of the hormonal equipment

Hypothyroidism increases susceptibility to certain infections; on the other hand, the injection of thyroid hormones considerably increases the resistance of rabbits to tuberculosis. The effect of diabetes and insulin[11] on the receptivity to gangrenous and suppurative infections is well known.

During recent years it has been noted that cortisone, a hormone that is secreted by the adrenal cortex, considerably decreases natural defence processes by slowing down the normal inflammatory reactions and the activity of the macrophages and of the reticulo-endothelial system. It follows that treatment with cortisone may produce an increase in the rate of multiplication of infecting micro-organisms and give rise to the appearance of latent infections.

ACTH, or the corticotrophic hormone of the pituitary, also favours the development of certain infections, but it is possible to neutralize its action by administration of another pituitary hormone—the somatotrophic hormone.

There exist in the tissues substances which are quite distinct from hormones, having an antibacterial activity and therefore contributing to the natural defence mechanism. Spermine, a substance which is active against the tubercle bacillus, has been isolated from the sperm and kidney of the bull. Certain lipid substances, which are able to attack pneumococci, have been extracted from the kidney and liver of rats and guinea-pigs, and it has been noted that the presence of these substances is related to the degree of resistance to pneumococci possessed by these animals.

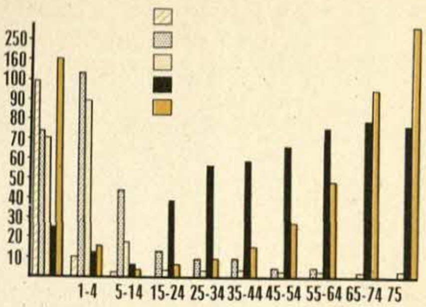

Fig. 4. Incidence of whooping cough, scarlet fever, diphtheria, tuberculosis and lobar pneumonia according to age. The diseases of childhood predominate during the first years; lobar pneumonia increases greatly with age. (Burrow, Textbook of Microbiology, *17th edition).*

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(10) Hormone: *a substance secreted in the blood by an organ and which stimulates the function of another organ.*

(11) Insulin: *a hormone secreted by the Islets of Langerhans of the pancreas.*

(12) Complement: *normal constituent of the blood and one of the humoral defence factors.*

(13) Malariotherapy: *treatment of nervous syphilis by means of an artificially produced malarial infection.*

The importance of diet

Diet and its vitamin content are of great importance in determining the degree of resistance of an organism to an infecting agent. Chronic starvation and vitamin deficiencies lower resistance and increase the host's susceptibility, particularly to tuberculosis. Deficiencies in vitamins C (causing scurvy), D (rickets), PP (pellagra) and B_1 (beriberi) were for a long time regarded as true infectious diseases. Micro-organisms were easily found in subjects affected by these avitaminoses because their normal defences were considerably reduced through the vitamin deficiency, increasing their susceptibility to ordinary infecting agents. Vitamin A protects certain mucous membranes (conjunctiva, respiratory mucosae) against infections so well, that it has been called the anti-infectious vitamin; vitamin C stimulates phagocytic activity and controls the synthesis of complement[12] and antibodies; and it is thought that it also exercises an antitoxic activity against bacterial toxins.

An extreme example is to be seen in the case of birds, such as pigeons and chickens. Neither are susceptible to the anthrax bacillus or to *Trypanosoma brucei*, unless starved or deprived of vitamin B_1, in which case they will succumb to infection by these agents. Here there is not only a variation in the degree of resistance but a clear appearance of receptivity in a species of animal that is normally refractory.

However, a diet that provides for the physiological requirements of a healthy organism, in quality and in quantity, is not always helpful for the natural defence of the organism against infections. *Trypanosoma brucei* proliferates less actively in the blood of a starved rat than in that of a normally fed rat, and the vaccinia virus produces less severe lesions in undernourished rabbits—phenomena which are no doubt related to the requirement of these parasites for certain metabolites becoming more and more rare in the undernourished hosts.

The importance of temperature

It has been known for a long time that certain environmental factors (temperature, humidity) influence the degree of resistance. Certain infections (pneumonia, broncho-pneumonia, influenza and colds) are liable to occur more often in winter, when cold, together with humidity, helps them to take hold. It is also known that high temperatures can increase resistance to certain infections: attacks of fever produced artificially or appearing spontaneously have led, for instance, to the cure of gonococcal infections, and malariotherapy[13] has been much used in the treatment of nervous syphilis because it provokes attacks of fever. It has been noted that rabbits maintained in an environment at 35° are less receptive to *Treponema pallidum*, the agent of syphilis, than those which live in colder environments. The normal chicken is resistant to anthrax because its internal temperature—of 42°—is unfavourable to the growth of this bacillus. But if we take a chicken, treat it with the anthrax bacillus, and immerse its feet and part of its abdomen in iced water, the body temperature will fall, the bacillus will multiply and the chicken will develop anthrax. This experiment shows, incidentally, the simplicity which is characteristic of experiments carried out by a genius, in this case Pasteur, by whom the experiment was made in 1878.

Resistance is strongest when it occurs for an entire species; where this is not the case, the resistance will vary in strength with the strains, races, or individuals. For a given species or race or individual, natural resistance is determined by hereditary factors; in addition to these, external factors have to be considered and these vary in intensity according to the species and the individual.

Natural resistance is always the resultant of defence mechanisms, not all of which are known, some of which are non-specific, and some of which have a certain degree of specificity, so that there is established not an hereditary resistance to infection, but a *possibility* of defence that is more or less effective against different infecting agents or groups of agents.

Natural resistance interposes the first defence against an infecting agent, which may be either effective to the extent of preventing the appearance of the morbid process or of causing it to disappear. The function of natural resistance is all the more important in individuals who have not or only very slightly, used, the mechanisms which confer acquired immunity.

Natural resistance also explains why an infecting agent usually produces a more serious morbid form when it is diffused in the endemic state. Below a certain threshold of resistance, races and individuals disappear; those which survive are the most resistant and, as in the case of Webster's mice and Gowen's rats, their offspring are naturally more resistant.

HISTORICAL NOTES

1878
PASTEUR *showed that the chicken, which is resistant to the anthrax bacillus, becomes sensitive to it when its body temperature is depressed below the normal (42°C).*

1932
WEBSTER *showed that the variety in the degrees of resistance offered by mice to given infecting agents is linked with hereditary characters.*

1943
GOWEN *and his research team established that hereditary factors determine the resistance of the rat to* Salmonella typhi murium: *these factors increase phagocytic activity and the formation in leucocytes of enzymes which destroy ingested bacteria.*

1956
ALLISON *revealed a relationship between sickle-cell anaemia and malaria.*

Antibodies

Robert FASQUELLE

The problem of acquired immunity has been a preoccupation of man for a very long time. Epidemics of smallpox, more perhaps than any other affliction, used to result in innumerable deaths and to return at fairly regular intervals. Yet anyone who had once contracted the disease and survived it was spared in subsequent epidemics. This was the first known example of acquired immunity. It gave rise to the practice of 'variolation': a healthy person was artificially inoculated with the content of a pustule from someone with a mild attack of smallpox. This was intended to produce a mild form of the disease which would protect the inoculated person from a subsequent fatal attack. In 1796, instead of inoculation with smallpox, Jenner introduced inoculation with cowpox (vaccinia), a disease of the cow which he considered to be an attenuated form of smallpox. This vaccination had the enormous advantage that it was never dangerous, whereas variolation sometimes produced a fatal attack of smallpox in the inoculated individual and above all made him infectious to other people. It is remarkable to note that all these results were obtained while the existence of micro-organisms was unknown.

In other diseases, which we now term infectious, the state of acquired immunity escaped attention for a long time. The confusion which reigned in the study of 'anginal fevers' (throat infections) and 'enteric fevers' stood in the way of discovery. It was necessary to wait until the second half of the nineteenth century, when bacteriological techniques were made available by Pasteur and Koch and the cause and identity of many infections had been discovered, for the problem of acquired immunity to be approached seriously.

The first experiment was in fact carried out in 1880 by Pasteur with the germ that causes cholera in hens. In a bird not having previously contracted the disease, inoculation produces a fatal attack of the disease, but in a hen which has previously contracted and recovered from an attenuated form of the disease a further intramuscular inoculation of the virulent germ does not produce any pathological manifestation. In homage to Jenner, Pasteur retained the term 'vaccination', although in this case the causal micro-organism had been attenuated by artificial means in the laboratory. These observations did much to stimulate enquiry into the mechanism which determined this acquired immunity at will.

Soon, and almost simultaneously, three hypotheses were put forward. The first proposed that the host organism lacked some specific substance indispensable for the multiplication of the micro-organism (a situation analogous to a broth culture, where growth stops when all the nutritive elements have been consumed). The second attributed the acquired immunity to the accumulation in the host of substances produced by the germ, which subsequently hinder the development of the germ. In following up this second idea and trying to isolate inhibitory products, it was found that when the filtrate of a culture of the bacteria was injected into an uncontaminated hen it produced a transient dazed condition. This led the experimenters towards the third hypothesis of toxicosis: that a chemical compound produced by the germ may be responsible for the symptoms of the disease, at the same time being endowed with an immunizing property. In this connection an observation by Roux and Chamberland was particularly indicative. They found that the filtrate of a culture of 'vibrion septique' (Cl. septican) injected in small doses into an uncontaminated guinea-pig rendered the animal capable of resisting an otherwise lethal infection by the inoculation of the living bacterium.

It remained to be established how the immune animal differed from the unimmunized animal.

Antibodies and immunity

In 1889, Héricourt and Richet described the protective action (it was only slight) of serum from patients who had contracted a staphylococcal infection. Charin and Roger

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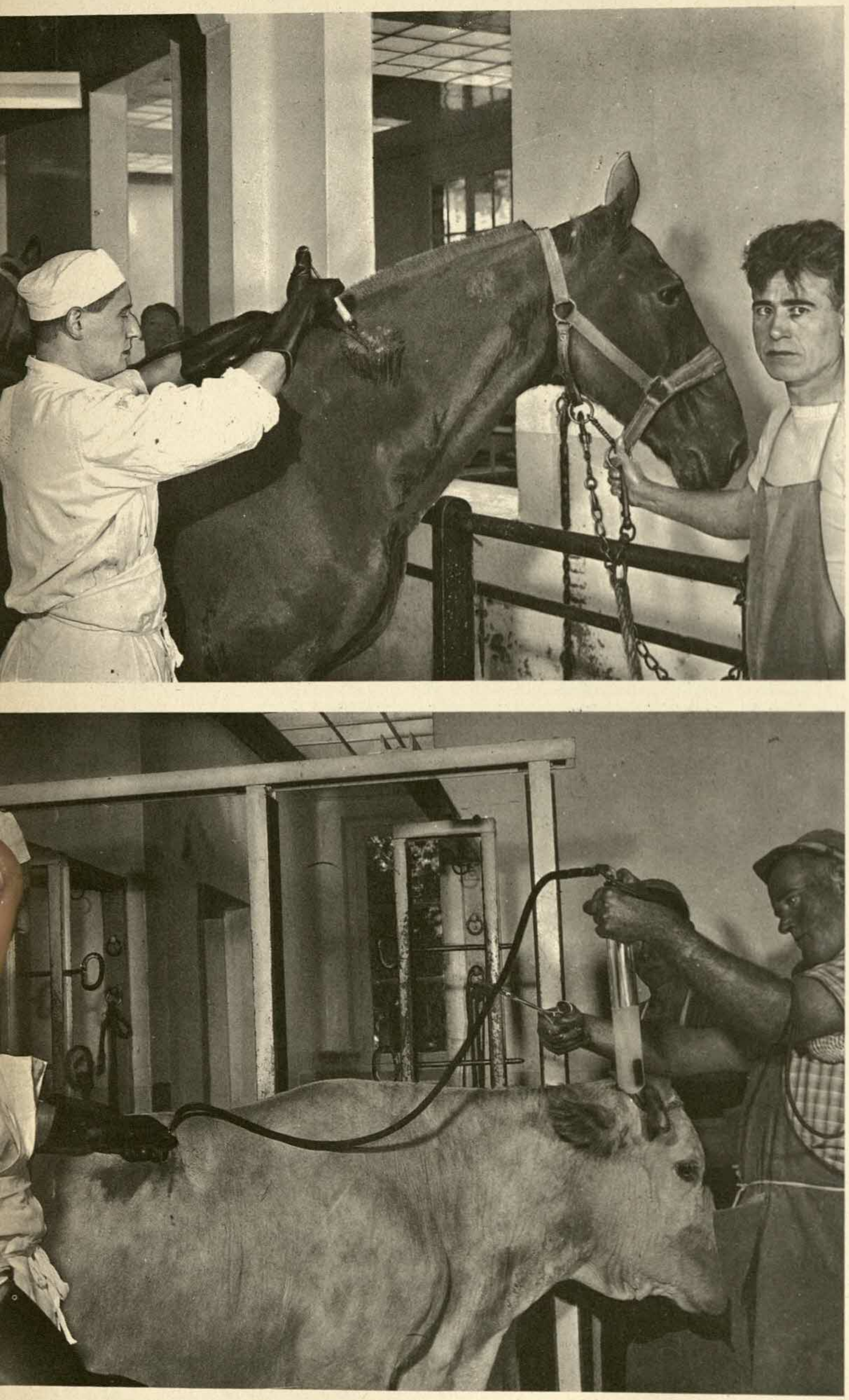
showed that pyocyanic bacilli grow in abnormally agglutinated colonies in a tube containing the serum of a rabbit previously 'prepared' by the injection of this germ. In 1890, Behring and Kitasato discovered the antitoxic power of serum from animals which had received small doses of diphtheria and tetanus toxins, and in 1896 Beclère, Chambon and Saint-Yves Ménard indicated the existence of an antivirulent power in the serum of heifers previously inoculated with the vaccinia virus.

The evident analogy between observations made in three different infectious diseases gave just cause for the generalization that, after an infection, soluble substances appear in the serum of the host organism which neutralize the pathogenicity of the germ. To these substances the name antibodies was given.

But antibodies were also found in the serum of animals not infected with micro-organisms or the products of micro-organisms. Calmette (1894) showed the presence of antibodies in the serum of animals which had been injected with poisons of animal origin. And in 1898, Bordet recognized haemolytic antibodies in the serum of animals which had been injected with red blood cells from other species of animal. The following year, Tchistovitch showed that, as in the case of red blood cells, the injection into an animal of a simple serum foreign to it also gives rise to antibodies which, mixed *in vitro* with the original injected serum, produce a precipitation.

As the name antibody had been given to all these different kinds of soluble substance that appeared newly in the serum of injected animals, another name, 'antigen', was given to all those substances (as dissimilar as micro-organism, toxin, snake venom or simple serum) with the common property of inducing the appearance of antibodies.

These mysterious soluble antibodies present in the serum have been subjected to chemical and physical analysis. They are precipitated by alcohol and ammonium sulphate under the same conditions as the normal serum globulins. On ultra-centrifugation they produce sediment in the same way as normal globulins. Electrophoratic diagrams show their range to be within the usual limits of the



The animals which provide the blood from which serum is prepared are subjected to anything from seven to twelve vaccinations. The vaccine causes antibodies to appear— 1,000 units per cubic cm. These are the 'police' of the host organism and, with the help of phagocytes, they oppose the entry of antigens.

normal globulins, the immune sera being characterized by an increase in the level of γ-globulins (the anti-toxins migrating, however, with the β-globulins). In brief, the antibodies are the serum globulins of the organism that has been treated with the antigen; they differ from normal globulins only in one characteristic, their specific affinity for this antigen.

The essential problem here was to define the conditions required for a substance to be antigenic. First, the characters common to all the antigens were considered. From the point of view of physics, the volume in which they occur plays an important role. A bacterium or a whole blood cell are better antigens than any extracts that can be made from them; colloids are antigens but crystalloids are not. Chemical studies produce similar evidence. Essentially, large protein molecules are antigenic and if lipids and polysaccharides sometimes play a part, it is in association with these protein structures. Landsteiner showed that new specificities are conferred on a protein by artificially conjugating it with various chemical groups; the name haptens has been given to these groups, which determine the specificity (for example, the polysaccharides bound to protein in the antigen molecule). To sum up, the antigen is a molecularly large body, the nucleus of which is usually of protein, and the specificity of which is determined by the spatial configuration of its peripheral hapten constituents.

For a substance such as the one whose properties have just been outlined, to be antigenic, two other conditions are essential, and these depend on the organism which receives it. First, the substance must come into direct contact with the internal medium of the host. If introduced by the digestive route, it is decomposed by enzymes and loses its structure and activity. If, on the other hand, it is injected into the peritoneum or carried by microorganisms (of which it forms the peripheral constituents) it arrives in the internal medium of the host directly. The second condition is that the substance must differ from the constituents of the organism itself.

Formation of antibodies

Obviously one should look for the site of formation of antibodies in the place where the conflict between the antigen and the organism arises. The reticuloendothial system and the lymphatic ganglia block foreign substances that have gained entry. According to some, it is the lymphocytes, plasmocytes, or plasmoblasts in the process of maturation that assume the essential role in the formation of antibodies.

As the quantity of antibody formed greatly exceeds the quantity of inducing antigen, one has to conclude that the mechanism for formation of antibodies must somehow conserve a record of the stimulus which the antigen has imparted to it.

Pauling postulated that the antibody is generated in the formative cells by a process of moulding normal globulins on the model of the antigen; the antibody globulins would therefore be normal globulins slightly modified in their structure. One could also suppose that, within the cells, some of the antigen is incorporated into the normal enzymes of the host; these enzymes, slightly modified, would then form globulins with the antibody property.

Antigen–antibody reactions

The manifestations of the reaction between antigen and antibody depend on the experimental conditions. The *neutralization reaction* is the most spectacular. It appears when the addition of the antibody to the antigen suppresses either its toxic power (neutralization of toxin by antitoxin) or its pathogenic power (neutralization of the cytopathogenic power of a virus by its specific antibody).

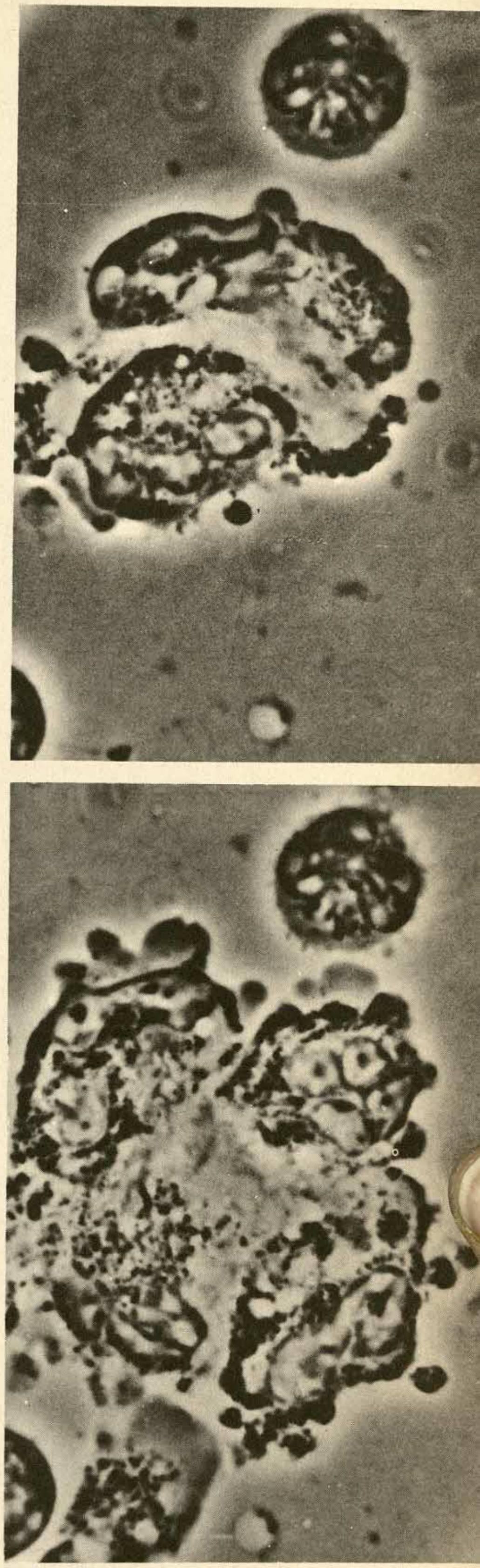
The *agglutination reaction* is one of the most commonly used: for example, the identification of a germ by sero-agglutination.

The *bacteriolytic reaction* is rarely used. It was Bordet who showed that in this case two substances were involved: the thermostable sensitizing agent (which is the acquired antibody) and the thermolabile non-specific complement (present in the serum of all uncontaminated animals).

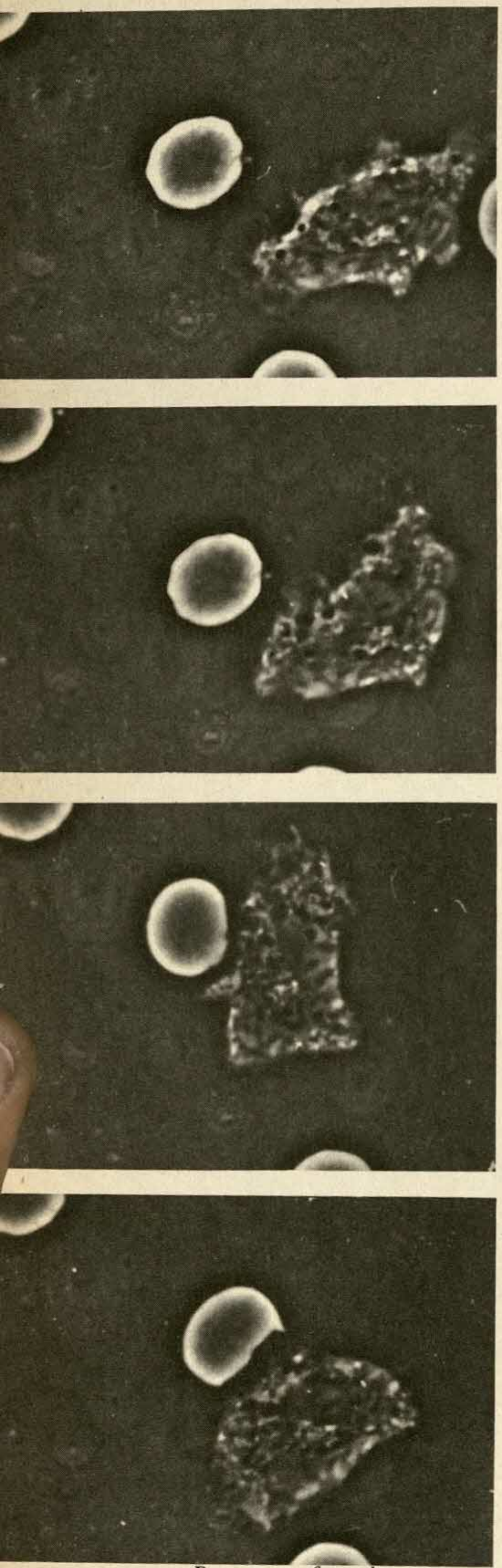
Since Bordet's discovery, the method of *complement fixation* has certainly become the most important immunologically; using a complex haemolytic system, it can show whether or not an antibody is present in a serum.

In the *opsonization reaction* the rapid engulfing and digestion of germs by polynuclear cells indicates that the germs have first been covered by the antibody and the complement.

The *precipitation reaction*, already mentioned, has been the basis of studies of considerable importance. From the theoretical point of view, this reaction has permitted analysis of the zone phenomenon in the simplest possible way; it has above all given immunologists the opportunity to discuss the intimate mechanism of the antigen-antibody



Antibodies help the process of phagocytosis: here, polynuclear cells are seen attracted by chemotaxis towards a cell nucleus which has been sensitized by an antibody. (Photo Robineaux).



By means of a phase contrast film it is possible to observe all the stages of phagocytosis. Here one sees the adhesion of a staphylococcus to the surface of a white blood corpuscle in the presence of the staphylococcus antibody and complement.

reaction. The supporters of the network theory maintain that a specific binding of a chemical nature occurs between multivalent antigen and antibody and this expands in every dimension until the volume of agglomerate formed is sufficient for precipitation. Another theory recognizes two phases in the precipitation: the first, specific and invisible, marks the entry into play of physico-chemical forces which are difficult to define. In the second phase the antigen-antibody complex now formed undergoes the flocculating action of electrolytes and then carries down in its precipitation a number of non-specific constituents of the antiserum.

From the practical point of view, the precipitation reaction is also of great importance. Even in liquid media, the existence of an antibody can be detected when a ring of precipitation appears at the interface of the antigen and the serum. Nowadays, the detection of lines of precipitation in gel media between two points from which antigens and antibodies diffuse in opposite gradients (Oudin's method), or Grabar's technique of immuno-electrophoresis, provide means of extreme sensitivity for the detection of antibodies which previously passed unnoticed.

Here we have indicated only the essential reactions. Others include the absorption of antigens on blood cells, Coombs's antiglobulin test, passive cutaneous anaphylaxis, and inhibition of viral haemagglutinins. More and more are being discovered.

This progressive increase in the number of methods for detection of antibodies has given certain immunologists such self-confidence that even when an antibody has not been detected they do not hesitate to affirm its existence, leaving it to be confirmed as technical knowledge progresses.

Antibodies and immunity

On the basis of the preceding facts an immunological theory of acquired immunity could be formulated thus: during the course of an infectious disease the host forms antibodies against the causal micro-organism, and when the level of these antibodies reaches a certain threshold level the subject recovers; the condition of immunity actively acquired in this way persists as long as the level of the antibodies remains high enough.

Instead of allowing an organism to prepare its own antibodies, one can passively transfer to it antibodies which have been elaborated against a given antigen by another individual. This immunity is acquired by passive means and is brought about by serum-therapy. It is an immediate immunity, which is an advantage, but it is also a borrowed immunity, since it is due to the globulins of another animal which has made the necessary effort to produce them as antibodies. It is also a transitory immunity, for these exogenous antibodies are rapidly eliminated and cannot be reinforced by further injections of antibody, because these antibodies are foreign globulins and are considered by the organism as antigens. The organism will form antibodies against them and here the risk of anophylactic accident arises.

Vaccination, on the other hand, consists of injecting into the organism to be protected an antigen which, without producing the disease, will enable the organism to form protective antibodies: in this case it is an immunity acquired actively, but at smaller cost. This immunity establishes itself after a certain delay necessary for the organism to form its antibodies. It is a personal immunity, being due to the individual's own globulins and for this reason it is lasting and can be strengthened by recurrent injections of the antigen, bringing into action the anamnestic reaction.

This reaction depends on the fact that an organism which has been in contact with an antigen in the past retains the ability to make the specific antibodies more quickly and in greater quantity when, at a later date, it is again stimulated by the same antigen.

The enzymatic theory of the formation of antibodies perhaps allows the finding of a physiological analogy between this anamnestic reaction and the fact, well known in microbiology, that a strain of bacteria accustomed in the past to grow in the presence of a certain substrate, acquires the ability to attack this substrate more rapidly than another strain which previously has never been in contact with it.

In toxigenic diseases, that is, infectious diseases which result from the effect of a toxin produced by the bacterium, the role of antibodies is clearly shown. This is the case in diphtheria, caused by *Corynebacterium diphtheriae*. The evidence is provided by the Schick reaction, which consists of the intradermal injection of a small known dose of toxin into an individual; this produces a positive erythrematous reaction if the serum does not contain the antitoxin, a negative reaction if it does. In an individual who has the disease, the reaction which was positive before becomes and remains negative. Serum-therapy will produce a negative Schick reaction, but this is only transitory. Vaccination by injection of anatoxin (three injections at fifteen-day intervals) produces a negative Schick reaction and this can be maintained by further injections of anatoxin.

The role of antibodies is also extremely important in tetanus. Our knowledge of diphtheria and tetanus, as far as physiopathology is concerned, is based principally on the work of Ramon (phenomenon of initial flocculation, transformation of toxins in anatoxins). In agreement with the theory, immunity in infectious toxigenic diseases appears to depend on the specific antibody, that is, the antitoxin. On the other hand, in other infectious diseases, such as whooping cough and typhoid fever, it is not the same.

Whooping cough is due to the Bordet-Gengou bacillus, which confers immunity. A form of vaccination exists, consisting of an injection of Bordet-Gengou bacilli killed after culture under special conditions (culture of germs in phase I). By injecting serum of human subjects who have been hyperimmunized (that is to say, who have received many injections of vaccine), it is possible to confer a fleeting passive immunity on a child. But no one has succeeded in isolating a specific antigen, a toxin, which can produce the disease experimentally—or the corresponding specific antibody. The nearest to a specific antibody that is known is an opsonin, present in the serum of persons who have had the disease, which favours the *in vitro* ingestion of the Bordet-Gangou bacilli by polynuclear cells. However, this opsonin disappears rapidly, but the active immunity lasts a lifetime.

There are three different points of view on this subject: the physicians admit that the mechanism of immunity in whooping cough is unknown; the immunologists, perhaps too confidently, proclaim their certainty that the day will come when perfection of their techniques will enable them to discover a specific toxin and antitoxin; and certain physiologists suggest that non-specific reactions play a part. It is also possible that some specific immunity mechanism is concerned which is due to something other than an antibody.

In the typhoid fevers (produced by *Salmonella typhi* or by *S. paratyphi A* or *B*), the disease confers a specific immunity. Vaccination by killed germs is effective, but injection of the specific antiserum is without any protective effect against the disease. The known antibodies are those which are specifically able to agglutinate the germs. However, the level of these antibodies is not proportional to the refractory state; the antibodies appear during the disease and disappear at convalescence, but the immunity persists.

Physicians and those who attach importance to the role of antibodies take two attitudes towards this problem, similar to those which they adopt towards whooping cough. The physiopathologists stick to the idea of a role of the neurovegetative system and of the reticuloendothelial tissue. They refer to the experiments of Reilly, which show that during typhoid fever the salmonellae are lysed in the lymphatic mesenteric ganglia, where they liberate their endotoxin. This endotoxin passes into the circulation and enters the diencophalic centres, thus provoking the stupor characteristic of the disease; and it also irritates the sympathetic nerve endings, producing swelling of Peyer's patches which may cause haemorrhages and perforations; finally, this sympathetic irritation provokes a state of alertness in the reticuloendothelial system, favouring the ingestion and destruction of salmonellae. From this the physiopathologists conclude that the immunity in typhoid fever is due above all to non-specific reactions. Obviously, it can be said that their explanation is insufficient, as the immunity itself is specific, operating against *S. typhi* or *S. paratyphi A* or *S. paratyphi B*, but not against all three germs. An explanation for this specific immunity remains to be found.

In the case of poliomyelitis the explanation of the specific immunity is presented in an apparently convincing way, but in reality not everything is clear. The antibodies in circulation neutralize the virus during its short phase of viraemia and prevent its fixation on the nerve cells, thus rendering the infection inapparent and limited to the digestive tract. Vaccination by the inactivated virus would be carried out in order to provoke formation of antibodies to a sufficiently high level. However, it is impossible to forget the deceptive results of specific serum-therapy even when used as a preventive measure.

In cowpox and smallpox the arguments against the exclusive role of antibodies in the formation of the condition of acquired immunity are even more serious than in the cases above. In these two diseases an *enzymatic* theory of immunity would be as acceptable as the so-called immunological theory; and the enzyme would account for the specificity of this immunity as well as—and perhaps even better than—the antibody.

Vaccination and serum-therapy

Hans SCHMIDT

The purpose of treatment with a vaccine or a therapeutic serum (a serum which is obtained from an immunized animal) is to raise the level of antibodies in the body fluids of the treated subject (a person or an animal). In the case of a vaccine it is the subject itself which forms the antibodies; in the case of a therapeutic serum, on the other hand, antibodies already prepared are brought to the organism. The inconvenience of a vaccine is that its action is slow, but it has the advantage of producing a protection that is lasting; exactly the opposite can be said of immune serum-therapy. Before the era of sulphonamides and antibiotics, vaccines and sera played a very important part in therapeutics. A few of them, however, have recently lost their interest, but vaccines such as anatoxins or the antipoliomyelitis vaccine, and antitoxic sera such as the antidiphtheria and antitetanus sera, are still in universal use.

All infectious disease presupposes an infection; that is to say, a penetration and reproduction of the microbe within the host, in spite of the natural defence which the skin and mucous membrane of the host provide. Some micro-organisms can act locally at their point of entry; others are carried by lymphatic, blood or nervous routes to different parts of the body, where they find conditions more favourable for their multiplication.

This process of the infection should be distinguished from an invasion of an organism by intestinal parasties (such as tapeworms) and from a contamination caused by, for example, a fungus.

An infection does not necessarily produce an infectious disease, as when the infecting agent is unable to withstand the defensive mechanisms which oppose it, namely, the bactericidal activity of body fluids and phagocytosis. In most cases the microbes which penetrate the body are destroyed; if this happens, they are called 'apathogenic' as a rule, and 'pathogenic'—but not always—if it occurs frequently. On the other hand, when a germ shows that it is capable of overcoming the natural defensive mechanisms, it is said to be 'pathogenic', that is, capable of producing a disease. In order to defend itself against pathogenic germs and to kill the germs which have already penetrated, the organism needs, as we have already seen, specific antibodies to be produced, the formation of which requires an incubation. Frequently, after the formation of the antibodies, the attack against the provocative agent coincides with the start of infectious disease that is clinically apparent. This can end by autosterilization, followed by a condition of immunity of varying degree and duration.

Sometimes the infectious agents remain in the organism for a long time as germ-carrying individuals—'carriers'. Sometimes the agent causing the infection is hidden in some part of the organism, for example, in the lymphatic ganglia. The outcome may also be death, always caused by toxic substances liberated by the micro-organisms.

The pathogenic power of bacteria can depend on a number of different properties; these may be based on:

1. capacity for unlimited reproduction in the body fluids (bacteremia), as in anthrax;
2. high capacity for reproduction of bacteria which produce endotoxins but which may also partly produce exotoxins (as in typhus);
3. relatively feeble reproductive capacity, but with formation of exotoxins, as in diphtheria, tetanus, botulism and gangrene;

(1) Anatoxin: *microbial toxin which has lost its toxic properties but not its immunizing properties.*

4. ability to cause intense localized inflammation (phlegmasia) associated with the danger of invasion of the blood stream by streptococci and staphylococci, with a simultaneous formation of toxins, as in scarlet fever;

5. capacity for the chronic destruction of tissues, accompanied by allergic reactions, as in tuberculosis and syphilis.

For many pathogenic agents toxic substances play a decisive role involving either endotoxins or exotoxins. Endotoxins are directly associated with the bacteria themselves but may also become effective after the death of the bacteria; consequently, our defence against them should consist firstly in the destruction of the germs. The most effective measure the host can take against the exotoxin-producing microbes, on the other hand, is the production of the corresponding antitoxins against the soluble toxin.

Preventive vaccination

The word vaccine was introduced by Jenner to indicate preventive (prophylactic) vaccination against smallpox with pustular liquid from a cow attacked by cowpox. Today the word vaccine is applied to the preparation made from living or dead germs used for inoculation. The name is also frequently given, erroneously, to preparations made from attenuated toxins (toxoids).

A vaccine holds substances called 'adjuvants' or activators. Ramon, in 1931, recognized the importance of these adjuvants for the preparation of antitoxic sera. By the inflammatory reaction which they produce, the adjuvants stimulate the host to a more intensive formation of antibodies. Ramon suggested the use of tapioca for this purpose. Starting from similar considerations, Glenny recommended the use of anatoxins (formolized toxins) precipitated by potassium alum; Linderström-Lang and Schmidt adsorbed the anatoxins by means of $AL(OH)_3$; Holt and Bousfield used aluminium phosphate, and Ross salmine sulphate. The adjuvants most commonly used today in vaccines for man are alum and aluminium hydroxide.

Adjuvants are unquestionably of value for the satisfactory formulation of antibodies. Glenny and Sudmersen established, in 1921, that it is as important to know the intervals between injections of vaccines as it is to know the concentration of the activator, which has optimal efficiency when the adsorption has reached the saturation point.

The first injection should be sufficient to stimulate the formation of antibodies, creating in this way a basic immunity. The second injection is made at the end of an interval, which should not be too short; in mass vaccinations this interval is four weeks. A longer interval of two to three months or more, particularly in the case of antitetanus vaccination, frequently gives rise to the appearance of a higher level of antitoxin in the blood.

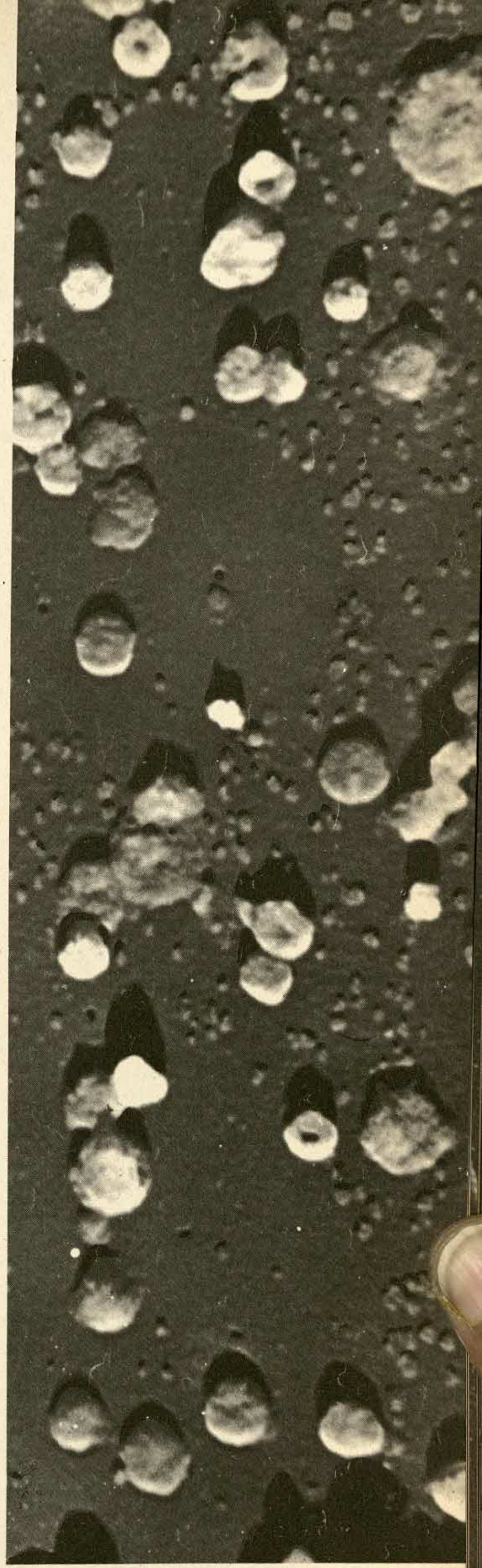
Preventive vaccination, therefore, normally consists of two injections. However, during an epidemic or when there is an increased possibility of infection (for example, by the tetanus bacillus in wartime), a supplementary 'booster' injection is recommended. As far as the duration of the effective immunity is concerned, it is not the level of antibodies in the circulating blood that is important, but the state of the cells which enables them to respond immediately to the excitation produced by the new injection; this favourable state enables rapid liberation of antibodies already formed, and fresh production of antibodies in large quantities.

There is considerable evidence to suggest that once acquired, the disposition of an organism to elaborate antibodies lasts as long as the antigenic molecules remain in the cells (Haurowitz, 1949); however, the real mechanism for the formation of antibodies is not yet perfectly understood.

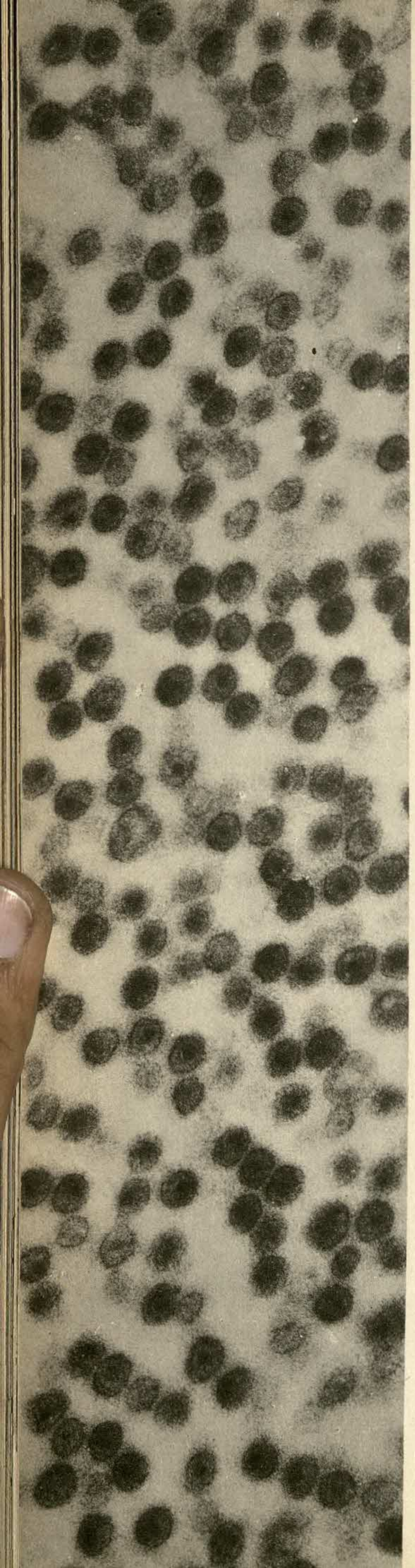
Different types of vaccine

Following the increasing success of chemotherapeutic and antibiotic treatments, prophylaxis by vaccination with dead or living germs (in the latter case, germs with attenuated virulence) has certainly become less important. It retains its value, however, for mass vaccinations, for example, against typhoid fever, cholera, plague and dysentery, where it is not possible to ensure effective immunity by means of chemical substances. The agents responsible for these diseases contain endotoxins. The object of vaccination is to produce antibodies against these endotoxins which *in vivo* will increase the bactericidal power of the blood.

This actively acquired immunity has shown itself to be extremely important. People who are sufficiently vaccinated are certainly not entirely protected against disease; they get it in a benign form and so do not die from it. However, the immunity is less long-lasting than that obtained against exotoxins liberated by diphtheria and tetanus germs; it also has the inconvenience of often producing local or



Newcastle virus, of various sizes. (Mag. × 50,000. Istituto Superiore di Sanità, Rome).



general reactions, particularly in the case of preventive vaccinations against typhoid fever and dysentery. It should be considered whether or not it is preferable, in these cases, to replace vaccination by a combination of phages and antibiotics taken orally and special vaccine therapeutics.

Preventive anti-cholera vaccination is based on the same principle, but in order to prepare a useful vaccine, it is necessary to take into account the locally different strains of bacteria. Against plague it is preferable to use a vaccine prepared with living bacilli, but bacilli with attenuated virulence. The greater efficiency of the living vaccine is attributed to the protein constituent of the endotoxin which is not broken up as a result of killing the bacteria through heat.

Prophylactic vaccination against staphylococci and streptococci is ineffective; a therapeutic vaccination, on the other hand, is indicated in cases for eventual autovaccination and especially where resistance of the cocci to chemotherapeutic agents has developed.

Serum-therapy

So far we have considered vaccination principally as a prophylactic technique. It is thus that Jenner conceived anti-smallpox vaccination and Pasteur anti-rabies vaccination. The latter is carried out with spinal cord of infected rabbits, although now a vaccine made from the killed virus is also used (Hempt). Vaccination against rabies is legitimate because in most cases, depending on the position of the dog bite, the period of incubation is very long and the organism has sufficient time to produce the necessary antibodies. Therefore, in most infectious diseases, vaccination is no longer opportune. The patient needs preformed antibodies immediately, such as those present only in the serum of convalescents or of animals immunized by many previous vaccinations.

Serum-therapy, introduced by Behring and Kitasato (1890), was originally of value in antitoxic therapy against tetanus and diphtheria toxins. The neutralization of these exotoxins is sufficient to produce a cure without having to kill the responsible bacilli; the destruction of the bacilli is left to the defensive forces of the organism. In order to obtain the toxin produced by the diphtheria (Roux, 1887) and tetanus (Kitasato, 1890) bacilli, the bacteria were cultivated in broth and the filtrate of the culture which contained the toxin was used for vaccination. Many microbiologists are now trying to obtain highly purified toxins (Pope, Relyveld and Raynaud, Lapresle, Poulik); however, so far a toxin has not been prepared in a sufficiently purified state to allow detailed chemical analysis and the establishment of its structure.

In the early days, the use of toxins was not without danger for the animals used; consequently, at the beginning of an immunization very small doses had to be given, and the treatment lasted for an inconveniently long time. Later, when the toxin could be transformed into anatoxin[1] (toxoid), the treatment took less time and was highly beneficial. In the early 1920s Löwenstein, Glenny and Ramon, quite independently, succeeded in rendering toxins harmless by treatment with formaldehyde at about 37-50°. Besides horses, oxen, mules and sheep are also vaccinated against diphtheria and tetanus in order to provide sera of different origin for treatment of patients who might be allergic to the serum of one of these animals.

Advantages and disadvantages of antitoxic sera

In antitoxic sera, the specific antibodies (antitoxins) are bound to certain globulins which can be isolated and concentrated by appropriate procedures whereby the serum becomes better stabilized. All the commercial antitoxins, the activity of which is expressed in Ehrlich[2] units, are no longer crude sera but have been purified; that is to say, the greater part of their inactive proteins has been removed. From the therapeutic point of view. injection of purified sera immediately produces an antitoxic effect and it is very important to determine the best time to inject and the optimum quantity of serum to use. The toxins will become attached to cells; thereafter, they cannot be neutralized by the antitoxin, or at least, much less easily than the toxins still circulating in the blood.

Antitoxic serum-therapy has certain disadvantages. As an antiserum is a substance

Medicine uses vaccination against viruses. Spectacular results have been obtained for poliomyelitis, influenza and other diseases. Can we hope that one day it may be possible to vaccinate against certain cancers, due to viruses, such as this one, which is responsible for leukaemia in the chicken? Research is being carried out with this purpose in view. (Electron micrograph, mag. ×56,000, by Dr W. Bernhard, of the Institute of Cancer Research, Villejuif, France).

foreign to the organism into which it is injected, the serum is rejected, whilst at the same time antibodies against the foreign proteins are formed. This causes hypersensitivity to a further injection of serum from an animal of the same species, an effect which the physician has always to bear in mind. Thus, the passive antitoxic protection never lasts long and to a certain extent hinders the formation of active antitoxin in the organism itself; after anti-diphtheria and anti-tetanus therapy patients are not sufficiently protected against another attack by the pathogenic germ.

Formerly, anti-diphtheria serum was employed to protect people living with or associated with the patient; anti-tetanus serum was injected in the wounded as a protective measure. Today, prophylaxis against diphtheria and tetanus by means of anatoxins has become so widely used that classic diphtheria is only rarely observed. Similarly, in the Second World War, soldiers who had been sufficiently vaccinated did not suffer from tetanus. Although we now possess antibiotics efficient against diphtheria and tetanus, the use of serum-therapy remains necessary, for the neutralization of toxins already formed can be achieved only by the use of the antitoxic serum.

Antitoxic sera have been prepared against other diseases, such as botulism, in which the activity of bacterial exotoxins is involved. The antiserum for this disease must take into account at least three different species of anaerobic bacteria which penetrate into the body and form toxins. An active protection against gas gangrene by injection of the antitoxin is possible at least in principle. In the same way, a serum has been prepared to overcome the poisoning produced by eating *Amanita phalloïdes* (the 'Death Cap' toadstool). The antitoxin against the amanita toxin is difficult to prepare and is not produced commercially.

Certain enterobacteria (Gram-negative germs) which contain endotoxins also produce exotoxins, for example, dysentery bacilli of the Shiga type, certain colon bacilli (peritonitis), typhus and cholera bacilli. The use of antitoxic sera against some of these bacilli has been proposed, but the idea has lost much of its importance because of the treatment with antibiotics, chemotherapy or phages.

We must mention here diseases due to staphylococci and streptococci. There are strains of staphylococci which produce more and more frequently an intestinal intoxication by an exotoxin and this intoxication requires an antitoxic serum. Certain streptococci are considered to be the agents responsible for scarlet fever (Dick) through the mediation of phage. It is known that normally non-toxigenic diphtheria bacilli, after having entered into symbiosis with certain phages, can develop into toxigenic strains. In the same way, scarlet fever is thought to result from the combined infection by a virus and streptococci, the bacteria, after symbiosis with the virus, producing the scarlet fever toxin. It is found that neutralization by the antitoxin is therapeutically most effective in cases of virulent scarlet fever.

Vaccination against viruses

It is more difficult to predict whether vaccination against any particular virus infection can be effective, for the antibodies present in the blood and lymph, unlike the virus, do not penetrate within the cells. Nevertheless, prophylactic vaccination by living or dead viruses has been shown to be very valuable in diseases such as poliomyelitis and influenza. In these cases, the humoral antibodies link themselves to the virus before it penetrates the cell membrane. On the other hand, treatment with serum from convalescents has been shown to be equally useful. A preventive vaccination against diseases produced by viruses can be carried out, not without running certain risks, using a virulent virus, as Jenner did against smallpox. Now that the artificial selection of viruses in cell cultures has become possible, dead or living viruses adapted to animal cells are used (in the case of living viruses, their virulence has been attenuated).

The use of therapeutic serum against diseases caused by viruses seems to be indicated only as an anti-toxic remedy. Viral exotoxins have not yet been discovered and so one can employ only serum of convalescents. What has been said about viruses is also applicable to Rickettsia,[3] the agents of exanthematous typhus. Rickettsia are rather similar to bacteria but, like viruses, they can live within cells. The agglutination test of Weil-Felix (agglutination of *Proteus × 19* by the serum of patients),[4] which is a valuable diagnostic aid for exanthematous typhus, is based on an antigenic similarity between *Proteus* and Rickettsia. Weigl (1921) has prepared an antirickettsia vaccine from the intestine of artificially infected lice. The preventive vaccination against exanthematous typhus will regain importance when lice, which act as carriers of the disease, become resistant to external insecticides (DDT).

(2) Ehrlich (1854–1915). *Nobel prizewinner in medicine. He established an arbitrary system for the measurement of the action of toxins and antibodies.*

(3) Rickettsia: *pathogenic micro-organisms intermediate between bacteria and viruses.*

(4) Proteus × 19: *saprophytic micro-organisms of the soil and water.*

Anaphylaxis and allergy

Albert DELAUNAY

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An organism that has already made contact with a microbe generally profits from this association by increasing its natural resistance to the aggressor: in other words, it becomes immune. But this is not invariably so. Far from increasing, the natural resistance may in fact grow less; here we are dealing with those important phenomena referred to as anaphylaxis and allergy.

In general, the repeated exposure of an organism to a toxic substance, antigenic or otherwise, brings about an increased resistance in the organism. There develops a state of acquired immunity, or habituation.

But the opposite phenomenon may also occur, and a substance harmless when first encountered may become noxious to a subject which has already been exposed to its effects. Such a reaction is brought about by an increase in sensitivity.

To describe these reactions the terms anaphylaxis (opposed to protection) or allergy (modified reaction) are currently used.

Manifestations of anaphylaxis

In the laboratory it is relatively simple to induce these reactions. Take, for instance, a guinea-pig not previously injected, and inject into its vein a common protein, for example, 0·5 ml ovalbumin from a hen. Nothing abnormal happens. The protein is completely without any 'primary' toxicity. But now let us take another guinea-pig which has already received one or more injections (subcutaneous, intraperitoneal or intravenous) of the same protein, and let us make a further intravenous injection (0·5 ml). This time, not always, but generally, it will be seen that there is a severe reaction, a genuine state of shock (anaphylactic shock). The animal is agitated, it runs round in circles and it shows sudden jerks and dyspnoea. The sphincters relax, and death may occur in five to seven minutes. An autopsy shows very considerable distension of the lungs, the result of severe constriction of the bronchial tubes. Why this violent reaction, this unexpected death? One must conclude that the previous injections have sensitized the animal to the protein, and have conferred on this a 'secondary' toxicity.

A different reaction, also anaphylactic in nature, may appear in an animal locally without causing its death. This was discovered by Arthus in 1903. If a rabbit is given repeated subcutaneous injections of horse serum, the first injections produce only a feeble local reaction; at most there may be a little oedema. But later the intensity of this reaction increases. The oedema becomes considerable and develops a pseudophlegmonous character. Finally, there appears a blackish gangrenous patch at the place where a new injection was made several weeks after the first one.

Studies of experimentally-induced anaphylaxis have indicated, on the one hand, which animals are the most sensitive, and on the other, which are the substances most likely to induce anaphylaxis.

A state of anaphylaxis can easily be induced in the guinea-pig and the rabbit. Other species of animals, however, are rarely suitable for this kind of research; the rat, for instance, becomes sensitized to horse serum only with great difficulty.

To induce a state of anaphylaxis many different substances may be used: foreign sera, foreign cells, milk, white of egg, sperm, microbes and microbial extracts. Thus, in guinea-pigs which have first been injected with killed bacteria (for instance, pneumococci) one can induce an anaphylactic shock, and even bring about death, by intravenously injecting a substance isolated from these bacteria and dissolved in water: for example, a specific polysaccharide which itself is entirely devoid of any 'primary' toxicity. Similarly, injection of a bacterial polysaccharide into the skin of a rabbit immunized with the corresponding bacteria leads to a localized reaction of the Arthus type.

Anaphylaxis is specific

The state of anaphylaxis always requires a certain time to establish itself. Moreover, it is strictly specific; only the substance used to sensitize an organism (or a component of this substance) can subsequently induce the appearance of the phenomena of hypersensitivity. These characteristics remind one of a reaction of antibodies. In fact, all the work in this field has indicated that the manifestations of anaphylaxis, be they local or general, are the result of reactions occurring in the organism itself, between antigens (or the corresponding haptenes) and specific immunoglobulins. Two points, however, remain the subject of eager discussion among specialists; in which part of the organism does this reaction occur, and by what mechanism does it bring about the irregularities spoken of as anaphylactic?

The opinion seems to be prevalent that the antigen-antibody reaction occurs at the level of the cells carrying the antibodies attached to their surface. An experiment, first conducted by Schultz and Dale, appears to prove this conclusively. A piece of unstriped muscle tissue or a fragment of the intestine, or, better still, of uterine horn, is taken from a guinea-pig immunized against a particular protein, and immersed in a solution of physiological saline. The addition of even a trace of the antigenic protein to the saline bath brings about the immediate contraction of the muscle. That this phenomenon represents a true antigen-antibody reaction cannot be doubted, because the Schultz-Dale reaction is strictly specific. Only the protein that served as antigen will bring about the contraction of the smooth fibres. The fact that the antibodies are attached to the cell surface and not free, appears likely, seeing that a piece of normal tissue, if perfused for several hours with a saline solution containing the antibody corresponding to the antigenic protein (for instance, serum from an immunized rabbit or guinea-pig), and then washed, by means of perfusion, with saline solution only, reacts like the organ from an animal immunized directly by active or passive means.

But what happens at the time of the rough combination of the antigen with the attached antibody? How does this combination produce the anaphylactic irregularities? As yet, one can only formulate various hypotheses.

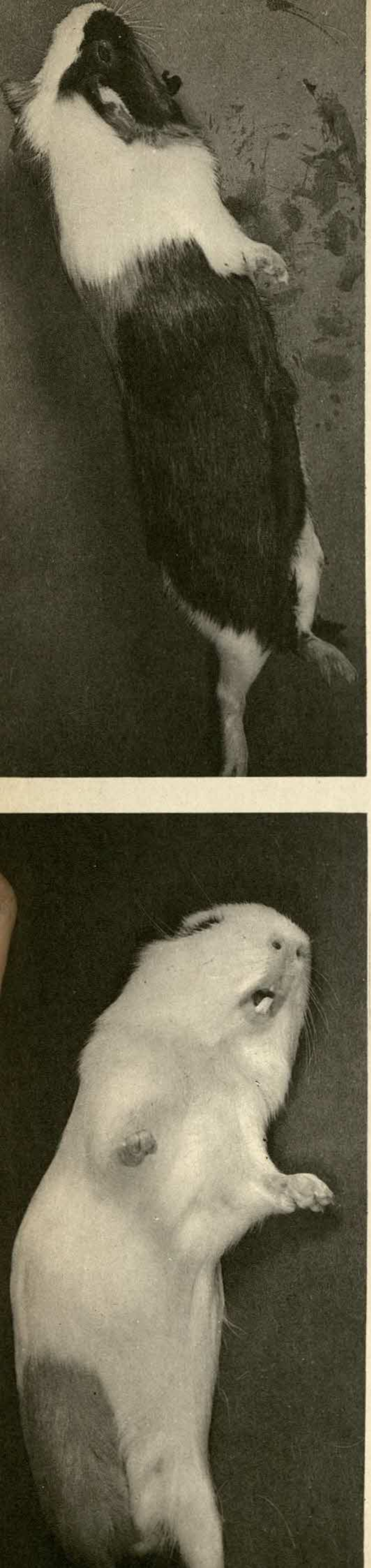
Many workers, following Dale and Lewis, put forward as causal agent a substance of the nature of a histamine. Such a substance would be suddenly released at the moment when the antigen combines with its antibody, and the anaphylactic reactions would be, in short, injuries of an histaminic nature. This is possible. At the same time, it must be remembered that the injurious effects of histamine and those of anaphylactic shock are not exactly analogous. In particular, histamine injected *in vivo* does not give rise to one characteristic symptom of anaphylactic shock: the loss of coagulability of the blood.

Man can develop hypersensitivity to all the antigenic substances he receives. Subjected, for instance, to repeated injections of a foreign serum (such as the serum from horses, which is frequently used in serotherapy), he may react with various abnormal manifestations which together constitute serum illness. Redness develops at the point of injection, and a painful tumefaction develops, with an itching, stinging eruption. This eruption frequently spreads and is accompanied by puffiness of the face, the eyelids and the tongue. Sometimes there are also coughing, heart spasms and albuminuria. The temperature rises to 38-9°C. Generally, these symptoms recede without leaving any trace. Some cases of death have been recorded, but these are exceptional.

Manifestations of allergy

If Koch bacilli are injected under the skin of a guinea-pig, one notices, a fortnight later, that at the point of inoculation a nodule has formed which finally gives rise to an ulceration (cold abscess) which persists, generally till the animal dies. Gradually, the infection spreads

Bottles of antidiphtheria vaccine in the drying-cabinet. (Photo Lod. Pasteur Institute, Garches).



to the regional lymphatic glands (adenitis), then to the other glands and the organs (spleen, liver, lungs, etc). The animal generally dies after several months.

Now, let us choose a guinea-pig already developing tuberculosis and inject it subcutaneously with a fresh dose of tubercle bacilli in a region distant from that where the first inoculation was made. One of two eventualities may then arise: either the animal will die within twenty-four hours (this occurs particularly if the primary infection started some time ago, or if a large quantity of germs was used); or the animal will survive. If it lives, there appears after two days an inflammatory and necrotic reaction at the point of re-inoculation, which then fades rapidly without giving rise to either a nodule or an abscess, or to any attack on the neighbouring lymphatic glands. This very peculiar behaviour was first noticed by Koch, and is for this reason frequently called the Koch phenomenon.

Essentially, it shows that an organism already infected with tuberculosis behaves quite differently from an uninfected organism when exposed to tubercle bacilli. It shows itself hypersensitive to substances produced by the microbe: in contact with them it reacts after a short delay; but at the same time, it seems to become endowed with a certain acquired immunity, because re-inoculation gives rise only to local lesions. This type of reaction, where phenomena of hypersensitivity are mingled with phenomena of resistance, is usually described as allergic.

Let us give another example, no less conclusive. Placing the virus of vaccinia on the skin of a fresh subject usually produces the following reactions. After four days, a small cutaneous lesion, a sort of enlarged pimple, appears locally, turning into a transparent opaline blister on the fifth day. On the seventh and eighth days the lesion acquires the appearance of a pustule, of variable size, with a pearly lustre, surrounded by a hard raised border. Then, from the twelfth day onwards involution begins. The pustule begins to dry, the crust turns brown and subsides during the third week, leaving a permanent scar.

These guinea-pigs have received several injections of a common protein. A little later, a new intravenous injection (0·5 ml) produces a severe reaction described as anaphylactic shock. The animal is agitated, undergoes convulsions and makes violent jerks. Death may occur in five to seven minutes. (Photo Lod. Pasteur Institute, Garches).

On re-vaccination the symptoms are very different. They are characterised by the earliness of their appearance, their intensity, and also by the rapidity of their development. In effect, one finds here what one saw in the animal re-infected with the Koch bacillus, and so may describe this also as a state of allergic reaction.

The term allergy, first proposed by von Pirket, has had a remarkable vogue. Like anaphylaxis—perhaps even more so—studies of allergy have been extraordinarily numerous. They have been made to discover, for instance, which are the allergic illnesses apart from tuberculosis and vaccinia. Several have been found: glanders, Malta fever, soft sore, etc. Some fungal diseases can also give rise to a state of allergy. In all these cases the hypersensitive state is easily detectable by the exaggeratedly vigorous reaction after cutaneous injection of an extract from the bacilli: tuberculin (for tuberculosis), mallein (for glanders), trichophytin (for infections due to a trichophyte), or of the germs themselves. By contrast, other illnesses induce little allergy, at least, according to the cutaneous injection test. These are illnesses caused by streptococci, pneumococci, and staphylococci.

But allergy phenomena do not only occur in infected individuals. They may also be found after repeated contact with the most varied substances. In fact, this latter form of allergy may be said to be the one most frequently encountered by doctors during the last few years. Very often, for instance, sensitization of an allergic kind appears to be caused by alimentary proteins. Further ingestion of this protein will cause gastro-intestinal disorders (vomiting and diarrhoea) and cutaneous troubles (nettle-rash, etc.). It may also happen that sensitization occurs via the respiratory passages; here epidermal particles from various animals are involved, also pollen-dust from plants or flowers.

Who has not met people who, to their great distress, find themselves sensitized to organic or mineral substances of great variety —materials used in industry, medicines (ipecacuanha, quinine, antipyrine, arsenicals, bismuth, pyramidon, sulfamides, etc), and people who react to contact with such substances by developing rashes, asthma, etc?

The substances that can induce allergies in man are innumerable. Nowadays, one can almost go so far as to believe that man, in certain cases, may become sensitized even to some of his own chemical constituents. This would be auto-allergy.

Cellular immunity to viruses: interferon

Alick ISAACS

The presence of specific antibodies in an organism is often sufficient to protect it against a virus, but there is no reason to think that antibodies are the only protection.

In addition to the antibody mechanism of immunity discussed previously, there is a second mechanism by which man and animals defend themselves against virus diseases, ie by the development of *cellular* immunity to virus infection. There are three main differences between antibody and cellular immunity:

(1) Antibody is produced by specialized antibody-producing cells, whereas cellular immunity is found in the cells which are actually infected by virus.

(2) Antibody acts by combining with and neutralizing the virus directly, whereas in cellular immunity the *cells* become resistant to virus invasion and there is no direct effect on the virus particles.

(3) Antibody is specific, eg antibody to poliomyelitis virus does not protect against yellow fever, and vice versa; cellular immunity is not specific and immune cells are immune to poliomyelitis, yellow fever and other viruses.

Cellular immunity seems to be due to the fact that the cells protect themselves by producing a substance called interferon, but before discussing interferon we must go back and look at a phenomenon from which interferon first emerged, the phenomenon of virus interference.

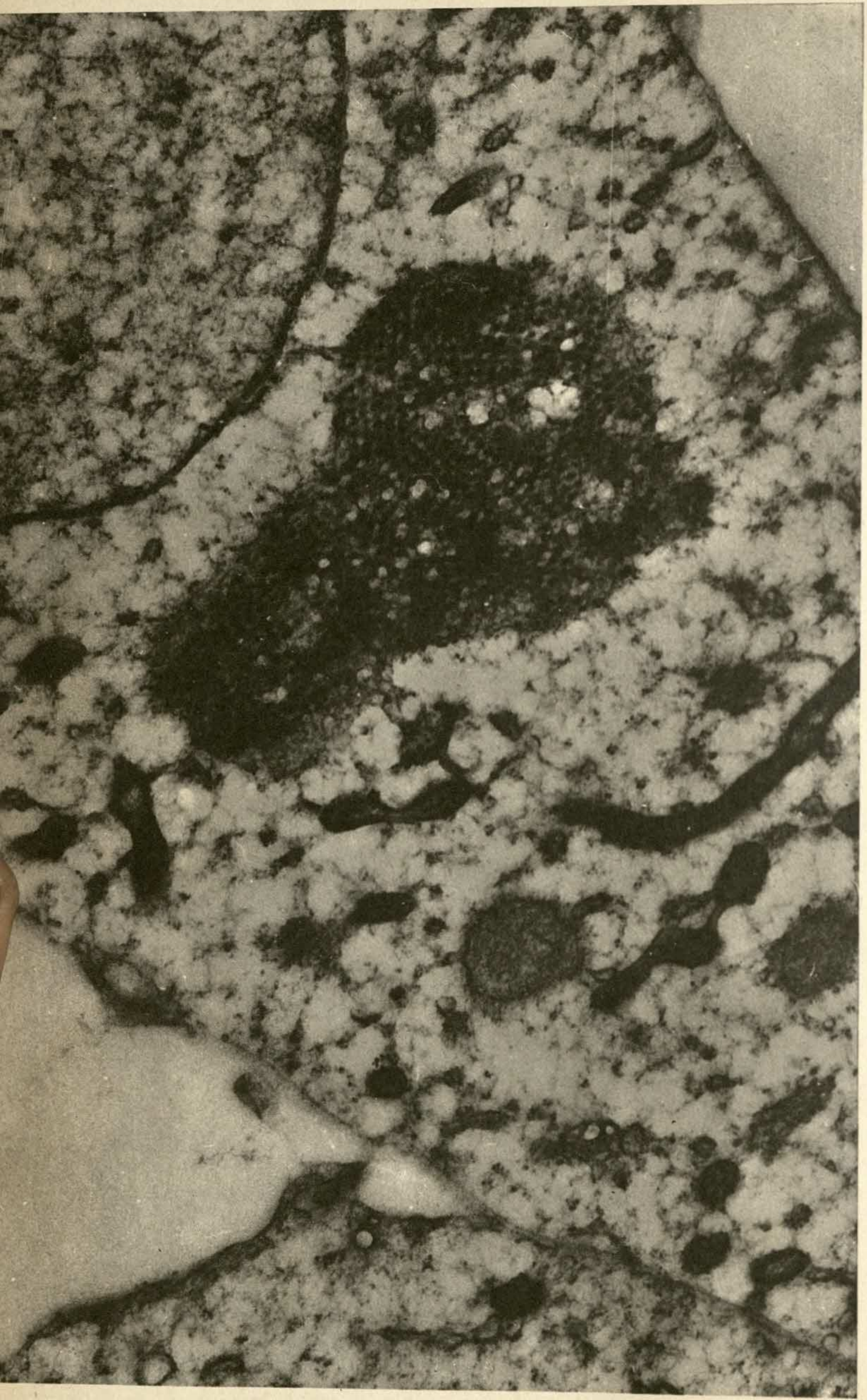
Virus interference

The first clear example of virus interference was the discovery, in 1937, that monkeys infected with the harmless Rift Valley fever virus were protected against the virulent yellow fever virus. These two viruses are quite unrelated to one another antigenically, so that this protection could not be due to the antibody mechanism described previously. Instead, the protection was attributed to virus interference; it was assumed that the first virus occupies cells and interferes with, or excludes, the second virus from growing in the same cells. After this demonstration many other examples of virus interference were described and it became accepted that when one virus occupies a cell it has the effect of excluding other viruses. But in what way is the second virus excluded? Is it unable to enter the cell? Experiments showed that this was not the case—the second virus was able to enter the cell but was unable to multiply within the cell, and it was assumed that the first virus blocks some key enzyme or uses up some nutriment which is essential for virus multiplication in the cell.

But, in 1957, it was found that the first virus, or interfering virus, induces the cells to make a substance which was called interferon, and which is capable of making fresh cells resistant to virus infection. The substance was found while the phenomenon of virus interference was being investigated, and because of this, it was given the name 'interferon'. But viral interference is a very academic phenomenon—a laboratory 'trick' which probably plays little part in natural virus infections of man and animals. Its interest for us is that it points the way to the understanding of cellular immunity to virus infection.

The properties of interferon

What is interferon? It is a protein of a molecular weight of about 65,000, ie, it is a medium-sized protein, smaller than antibody molecules. It can be produced in the laboratory by infecting cells from many different organs and animal species with many different types of virus. This suggests that interferon is produced by cells as a general response, perhaps as a defensive response, to the stimulus produced by virus infection. The interferons



induced by different viruses behave very much alike, but the interferons produced by different animal species show slight differences. This is not too surprising, since it is well-known that insulins, trypsins and antibodies from different animal species also show slight differences. The most important difference in interferons from different animal species is that they tend to show some species' specificity. For example, interferon prepared in chick cells will protect chick but not duck cells against virus infection, and the converse is equally true. However, the species' specificity is not absolute, and monkey interferon is quite active in cells of a number of different animal species, including those of man. An interesting discovery is that, following virus infection, interferon is liberated spontaneously from cells; this suggests that it may have the opportunity to protect cells surrounding those which were infected initially, and that it may therefore serve as a substance which will protect the whole animal from virus infection.

Interferon seems to be a quite stable substance and it can be kept in the refrigerator at 2-4°C for many weeks without significant loss of activity. It can also be stored frozen, or freeze-dried and it is possible to concentrate its activity very considerably either by freeze-drying or by other suitable methods. In 1960, considerable progress was made in purifying interferon by adsorbing it on to modified cellulose columns and then separating it under appropriate conditions of pH and salt concentration. By such methods and also by electrophoresis on starch gels, a considerable degree of purification has been achieved. From these results one can already see that interferon is a highly active substance and active preparations of interferon may contain as little as 5-10 micrograms (μg) of protein per cc.

Recovery from virus infections

The fact that interferon is formed in response to virus infection of cells in the test-

Cellular immunity is present in all infected cells. This gives the tissue an unspecific resistance against all viruses, such as poliomyelitis, yellow fever, etc. Some viruses, however, cause cancers without this general protection being conferred. The most recent research is concerned with those types of carcinogenic viruses that cause serious lesions. Cytoplasmic lesions are here shown in a cell invaded by REO III virus particles. The arrangement of these particles, in regular lines in the cell body, makes it easy to locate them. (Mag. × 20,000. Electron micrograph by P. Tournier).

tube suggests that it might also be formed during the course of virus infection in the animal body. This was shown to occur in rabbits inoculated with vaccinia virus, in chick embryos infected with influenza virus and mice whose lungs were also infected with it. The next question is what part interferon plays in recovery from virus infections.

For many years it was postulated that immunity to virus infections was largely, if not entirely, due to the formation of specific antibodies. The evidence on which this theory first rested came from a very neat experiment carried out in 1929 by Dr C. H. Andrewes. Dr Andrewes found that if he took tissues from normal animals he could infect them, in the test-tubes, with herpes virus (the virus which causes cold sores on our lips), whereas tissues from immune animals could not be so infected. Next he took tissues from immune animals, washed the tissues and kept them in the presence of serum from normal animals; conversely he took tissues from normal animals and kept them in the presence of serum from immune animals. When these tissues were infected it was found that the immunity was due to the serum, not the cells, presumably owing to the presence of antibody in the immune serum. Dr Andrewes was careful not to generalize from this experiment, but others who followed him have not always been so careful. The success of virus vaccines, such as those used in the prevention of poliomyelitis, has continued to focus attention on the antibody mechanism of immunity to virus diseases.

But while these results make it clear that antibody plays a vital role in preventing us from becoming infected, it is not clear that it is important in helping us to recover from our first infection with a virus. Indeed, some evidence has been accumulating that people with a rare disease (called hypogammaglobulinaemia) in which they are unable to make antibody normally, still recover from virus diseases in the same way as do normal individuals. These patients with hypogammaglobulinaemia are very susceptible to bacterial diseases, and before the days of antibiotics most of them probably died from some bacterial infection. Since it has been possible to save them with antibiotics one can study their reactions to virus diseases. Many of them have been shown to recover normally from measles, influenza, poliomyelitis and vaccination against smallpox without forming any significant amounts of antibody, and it seemed from this and other evidence that some other mechanism must be helping in recovery from virus infections.

This problem was studied by infecting mice with influenza virus by inhalation. The virus passes to their lungs, multiplies there, and sets up a pneumonia from which the animals may die or recover, depending on the dose of virus used. In these experiments, animals were killed at daily intervals and the amounts of virus, antibody and interferon were measured in their lungs and sera. Fig. 1 shows the method that was used to measure the antiviral action of interferon in the lung extracts.

The results of these experiments were that virus multiplied rapidly in the lungs and reached its peak by the second or third day. Large amounts of interferon were formed in the lungs, the highest concentration occurring about one day after the highest concentration of virus. By contrast, antibody was very late on the scene and was not detected until about one week after the concentration of virus had already begun to decrease.

This suggests that antibody may play a more important role in helping us to resist a second infection with a virus—the extreme rarity of second infections with such viruses as measles, poliomyelitis, yellow fever and small pox does not need to be stressed—but that interferon, appearing at the right place and the right time, may play a more important role in helping us to recover from a virus infection.

'Active and passive' cellular immunity

The discovery of antibody has been exploited in medicine in two different ways, by passive and active immunization. Passive immunization means that patients are treated by injecting them with pre-formed antibody in

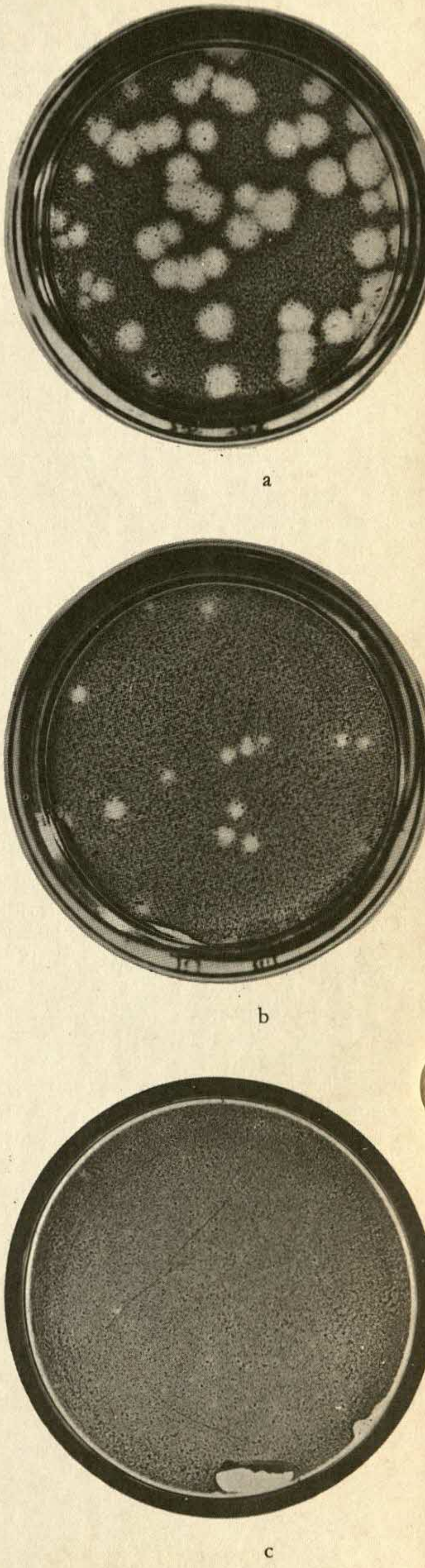


Fig. 1.

(a) This plate contains a layer of cells which has been inoculated with an encephalitis virus. Where the virus has attacked the cells, large holes (or plaques) appear.

(b) In this plate the cells have been treated with a small dose of interferon. The plaques are already fewer and smaller than those in (a).

(c) In this plate the cells have been treated with a large dose of interferon. There are no plaques and the cells are completely protected. (Photos A. Isaacs).

HISTORICAL NOTES

1937
G. M. FINDLAY and F. O. MacCALLUM found that monkeys inoculated with the harmless Rift Valley fever virus were protected against the virulent yellow fever virus. This is the first clear example of virus interference among animal viruses.

1957
A. ISAACS and F. LINDENMANN discovered interferon, a product of the viral interference phenomenon.

1959
Interferon was found to protect rabbits against epidermic infection due to vaccinia.

1960
D. C. BURKE reported substantial progress in the difficult task of purifying interferon.

1960
A. ISAACS and G. HITCHCOCK pointed to the role of interferon in the struggle against virus infection.

1960
Demonstration that interferon protected rabbits against the virus of vaccinia inoculated in the cornea (K. CANTELL and V. TOMMILA) and mice against fatal infection from one of the encephalitis viruses (G. HITCHCOCK and A. ISAACS). The latter workers also showed that mice could be protected against the same encephalitis virus by being actively stimulated to produce interferon.

order to supplement the antibody which they would normally form themselves. One example is the use of diphtheria antitoxin (prepared by injecting horses with diphtheria toxin) which is injected into diphtheria patients and gives considerable protection. Another example is the use of pooled human serum or gamma globulin (the fraction of serum which contains antibody) which is injected into children in order to attenuate or prevent an attack of measles. Active immunization, on the other hand, means that an individual is protected by stimulating him to produce his own antibody. The classical examples are the vaccination practised by the English physician, Jenner, to protect against smallpox, and the use by Pasteur of fixed rabies virus to immunize against rabies.

One could imagine that it might be possible, in similar fashion, to protect animals against virus infection either by passively administering interferon which had been preformed elsewhere, or by actively stimulating them to produce their own interferon. Both these mechanisms can in fact be made to operate. Passive protection by interferon has been shown in the case of rabbits injected in their skin or in the cornea of the eye with vaccinia virus, and in mice inoculated with one of the encephalitis viruses (Bunyamwera virus). The rabbit experiments were carried out by inoculating interferon and virus locally at the same site, and they serve to show merely that protection as seen in the test-tube can be repeated in the animal body. The experiments in mice are rather more ambitious, since in this case the interferon protected against infection by a virus which kills mice after invading the nervous system. Active protection was found when mice were injected with a harmless influenza virus which multiplies in the lungs and produces interferon at that site. The interferon passed out of the lungs and into the bloodstream and was able to protect a proportion of the mice against an otherwise fatal infection with the same encephalitis virus (Bunyamwera virus). In some ways this can be considered as merely another example of virus interference, in which the influenza virus protected the animals against the encephalitis virus. But there is a difference. In these experiments it was shown that the influenza virus remained in the animals' lungs, and the protection was due to interferon and not to the virus occupying cells and excluding second arrivals. It may, therefore, be possible to exploit the interferon mechanism, as it was possible to exploit the antibody mechanism and to protect man passively and actively against virus infections.

Evolutionary significance

Comparative studies have shown that the antibody mechanism developed at an early stage of evolution of the vertebrates. At present, similar information on the development of the interferon mechanism is not available. Nevertheless, we can speculate on the roles of antibody and interferon as mechanisms by which man has defended himself against virus infections.

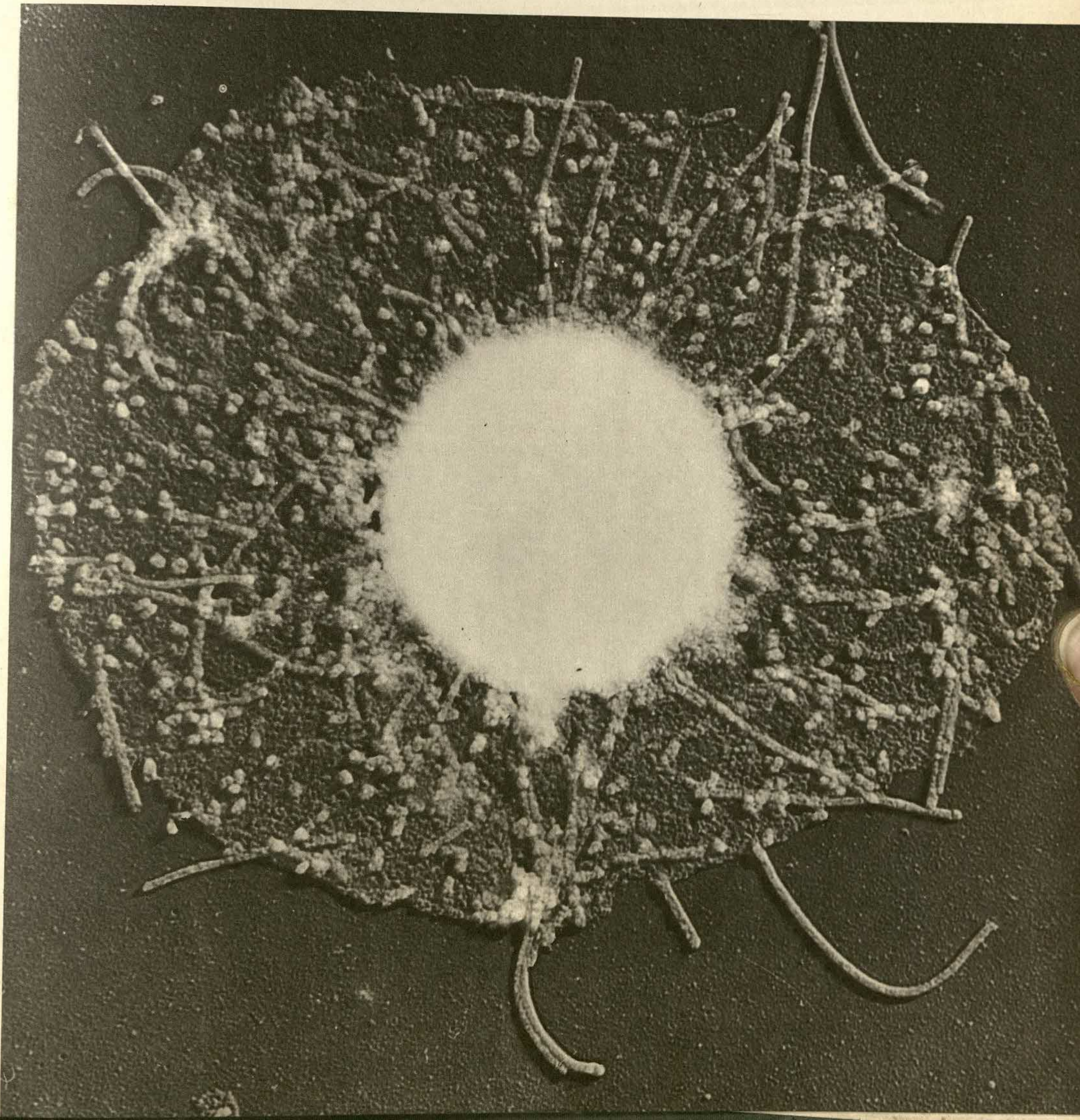
The interferon mechanism may have developed at a recent period in evolution as an adaptive response to superficial virus infections, such as those of the respiratory tract. This is not to suggest that interferon is a recently created product. It is more likely that it has some other function in the body, at which we can only guess dimly meanwhile, but that in response to particular virus infections it has become adapted as an antiviral substance which protects cells by preventing virus multiplication.

Practical considerations

Interferon was discovered too recently for it to be said whether it will prove to be of practical use in protecting man and animals against virus diseases, but there are many favourable signs. The problem is that viruses multiply in such intimate association with cells that any attempts made hitherto to find substances which would stop viruses from multiplying have run into the difficulty of toxicity—they have damaged the host cells along with the virus. The great advantage of interferon is that it is a natural antiviral substance, which is probably produced in our bodies in response to many virus infections and which seems to stop viruses from growing without harming our cells. A second advantage is that, at least in the test-tube, it is effective against a wide range of viruses, large and small and of different chemical composition. A third advantage is that it is not antigenic, that is to say that injection of interferon does not lead to the production of antibodies against interferon, so that there should be no barrier to its being used repeatedly. If it were antigenic it might be effective only once or twice and then its usefulness would be over. There are technical difficulties to be overcome before it could come into general use as an anti-viral substance, but it should not be beyond the ingenuity of man to overcome these difficulties.

PART FIVE

Man and microbes

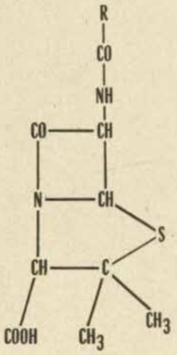


Newcastle virus (Mag. × 150,000. Istituto Superiore di Sanità, Rome).

The domestication of microbes

Nikolaï D. IEROUSSALIMSKY

Since ancient times, man has made use of microbial action for some of his industrial processes. However, the science of microbiology has advanced and old techniques have been perfected, empirical methods giving way to scientific. Today, all over the world, microbes of special kinds are carrying out innumerable chemical reactions faster and better than the most competent technicians.



The synthesis of penicillin. R represents any given radical; thus 100 different penicillins can be obtained.

Sometimes systematically, sometimes haphazardly, man is gradually subduing the living world and transforming it. But nature does not always submit; occasionally it hits back with what seems capricious revenge. Human industry and long practice have yielded some brilliant successes, among them the domestication of the species of animals that have supported our civilization, and the cultivation of useful plants.

This story is repeated in the conquest and use of micro-organisms. Some have reacted with hostility, not only to man but to his way of life, thus surpassing even savage nature by changing into pathogenic microbes in the human body. Others, by contrast, have become servants as obedient as the horse or the dog.

Fermentation, the first use of microbes

At first, man knew nothing of the existence of microscopic creatures, and the various stages in fermentation went on naturally, without his intervention. Grape-juice fermented spontaneously to produce wine by the action of yeasts occurring on the fruit surface. Acetic acid bacteria, in contact with wine, oxidized it, thus turning it into vinegar. Lactic acid bacteria curdled milk and, together with propionic acid bacteria, changed the curds into cheese. Other lactic acid types, living on the surface of vegetables, produced sour cabbage and cucumber. Yeast, occurring by chance in flour, fermented dough and the carbon dioxide evolved made the dough lighter.

In time, men induced fermentation by means of leaven obtained from the more successful corresponding food products. Unconsciously, they selected better types of micro-organisms, while the latter, at the same time, adapted themselves to the conditions around them.

The cultivated species of yeast can withstand up to 14-17 per cent ethyl alcohol, acetic acid bacteria up to 11 per cent acetic acid, and lactic acid bacteria up to 2-2·5 per cent free lactic acid and 10-12 per cent of lactates. On the other hand, such concentrations of acid or alcohol hinder the growth of any wild strains that gain access to the production cycle. Apparently, special defen-

The domestication of microbes, a modern biological study, has always been unconsciously practised by man. Wine, vinegar, and soured milk are familiar examples; also some cheeses which are intentionally inoculated with moulds to impart a distinctive quality to the cheese. On this piece of Roquefort, slightly enlarged, a fine mat of blue moulds can be seen in the centre.

sive substances (antibiotics) produced by some species of micro-organisms have a similar sterilizing effect. This is why microbial cultures have the property of auto-purification, ie the ability to eliminate foreign microflora.

Leavens often comprise two or three species of microbe adapted to living together in symbiosis. Rye-bread leaven contains special lactic acid bacteria, together with yeasts. Leavens with added bacteria are used to make butter and cheese.

The father of scientific microbiology, Pasteur, declared that the yeasts that bring about beer-fermentation would never be isolated from wild strains in nature. If this be so, what is the origin of such yeasts? Pasteur's reply was, 'I am very much inclined to believe that we have here a new example of plant or animal modifications which have become hereditary by prolonged domestication. We have not found wheat in the wild state; we do not know what was the first cereal; we no longer know the wild silkworm'. During the post-Pasteur period of microbiology, gradual use began to be made of pure cultures isolated from natural leavens, a technique introduced by E.-Ch. Hansen. The use of pure cultures made possible the stabilization of production processes and the avoidance of unforeseen mishaps.

The pure-culture method gave other advantages. It led to the isolation of different micro-organisms and the discovery among them of species forming new by-products. In this way arose the production of organic acids (lactic, butyric, citric, gluconic, fumaric), acetone, butyl and isopropyl alcohols, butylene glycol, sorbose, enzymic preparations (protease, amylase, pectinase), some vitamins (riboflavin, ergosterol, vitamin B_{12}, etc), and antibiotics.

The culture of micro-organisms

Antibiotics play a very important part in the fight against pathogenic microbes. Pasteur had already observed that when several species are grown together, some bacteria oppose the development of others. Metchnikoff suggested that the antagonism between lactic acid bacteria and saprophytic bacteria should be used for treating intestinal disorders. Thanks to the work of A. Fleming (1929), H. Florey, B. Chain and others extracted the first antibiotic, penicillin, in 1940 from cultures of a fungus, *Penicillium*. In 1944, after long, patient and systematic research, Waksman successfully extracted streptomycin from streptomycetes. In the next decade, by making use of various actinomycetes, chloramphenicol, aureomycin, neomycin, terramycin and other antibiotics were obtained. In fact, about a hundred different antibiotics are known, of which only thirty or forty have practical importance.

Some micro-organisms are used in animal nutrition, for example, the yeast preparations containing various essential proteins and vitamins. Some bacteria are employed in soil fertilization: nitragin, azotobacterin, etc. Cultures of bacteria and viruses with their virulence artificially attenuated give rise to vaccines and sera for medical use.

The micro-organisms recently introduced into industry have not yet had time to acclimatize or adapt themselves to industrial conditions. On the contrary, we are obliged to modify the technological environment to suit their natural requirements, and to protect them against invasion by foreign microflora.

Adaptation and mutation of micro-organisms

Left to themselves in nature, micro-organisms are subject to various adverse conditions, some of which are often very prolonged. This is why they are adapted for life in a wide variety of environments. Now, man not only uses some of the processes of microbes, but chooses an environment that will in general intensify such processes. Sometimes a small change in nutritive medium or culture conditions is enough to divert the biochemical activity of microbes into an entirely different direction; here lies the key for directing their living activity. For example, a species of bacterium can be made to carry out the most diverse oxidative reactions. The same mould may provide a given enzyme, some antibiotics, or some specified organic acid.

Because their activities are specialized, microbes gradually undergo adaptive modi-

Chemical industry obtains from bacterial products substances that are difficult or impossible to synthesize chemically. Several kinds of fungi are thus used in agriculture or medicine. Micro-organisms are also used for carrying out certain chemical reactions as stages in the synthesis of certain compounds: the bacterium Pseudomonas *(here magnified 20,000 times) is used in the synthesis of ascorbic acid (vitamin C). (By courtesy of the French National Institute of Agronomic Research).*

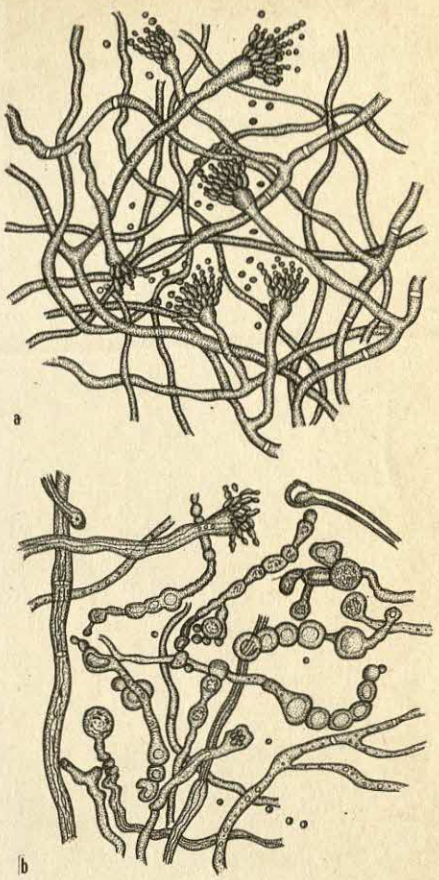
fications, to which microbiologists also contribute by systematic selection. After twenty or thirty years of productive activity, some micro-organisms can be clearly distinguished from their wild ancestors by their greater productive capacity; for example, the aspergillus-type fungi producing citric acid, and the bacterial acetone-butyric fermenters. Without doubt, microbial domestication is continuous and accelerating.

Nevertheless, microbiologists still find the rate of domestication too slow, and they sometimes need to bring various mutagenic agents into action: ultraviolet and radioactive radiation, mustard gas and other chemical reagents. These change the hereditary make-up (genotype) of the microbes, sometimes giving rise to mutants more active in producing valuable end-products. The early cultures of *Penicillium* produced no more than 50 units of penicillin per cubic cm, whereas the activity of some mutants now in existence reaches 9,000 units per cubic cm. Also in use are highly active mutants descended from organisms producing streptomycin and other antibiotics, and vitamin B_{12}. On the other hand, growth and other vital functions of mutants are often weakened. This is why careful attention is still needed, analogous to that bestowed on greenhouse flowers or thoroughbred domestic animals.

Microbes in the service of chemistry

Whereas the old microbiological processes, initiated at the dawn of human culture, were mainly used for food and drink, over the last ten or twenty years micro-organisms have acquired an increasing importance as producers of substances difficult or impossible to synthesize by pure chemistry. Such, for examples, are bacterial toxins, some enzymes and substances such as antibiotics and vitamin B_{12}, produced solely from micro-organisms. Pure cultures of phytopathogenic fungi of the species *Fusarium* produce gibberellins, compounds used for stimulating plant growth, and fungi of the type *Claviceps* make the alkaloids of rye ergot, used in gynaecology and therapy. In the production of glutamic acid, the bacteria-assisted method is better than the chemical synthesis. It is difficult to predict what other physiologically active substances microbes will yield, but our expectations will certainly not be in vain.

In other processes, micro-organisms are used to produce certain chemical reactions constituting single stages in the synthetic pathway towards the desired compounds. For example, bacteria of the types *Pseudomonas* and *Acetobacter* oxidize sorbitol to sorbose and glucose to 5-ketogluconic acid, products used in the synthesis of ascorbic acid (vitamin C). Because of their ability to act on one or two specific carbon atoms in the molecules of organic compounds, microbes, as specific reagents, are more sensitive and discriminating than chemical reagents and the usual catalysts. Their advantages are seen particularly clearly in the production of steroid hormones, when microbes help to achieve various conversions for which current chemical methods are not sufficiently precise. For example, they help in the synthesis of male and female sex hormones, as well as the cortico-adrenal hormones which have become very important in the treatment of rheumatism and other illnesses.

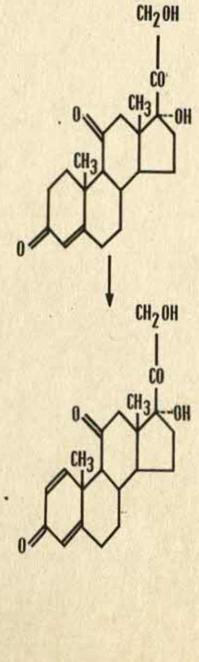


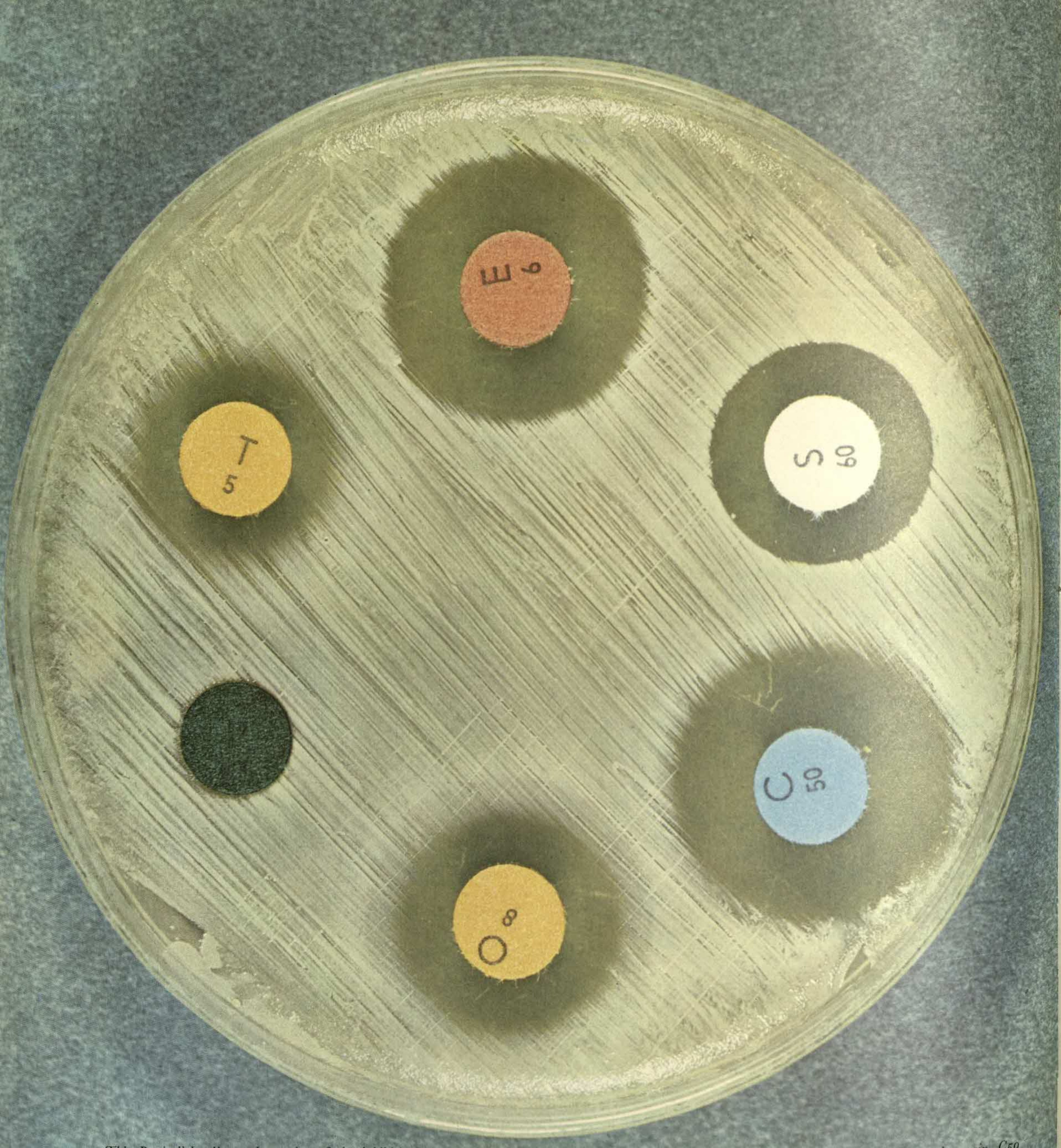
Aspergillus nidulans: *(a) original culture, (b) mutant obtained by radiation with ultra-violet light which increases the quantity of proteolytic enzymes (after S. Z. Brodska). This is a good example of microbiology applied to problems of the food industry.*

Some chemical products of micro-organisms are harmful to them. During the First World War, glycerine was obtained from yeast cultures by adding alkalis or sulphites, which hinder cell growth. Environmental conditions that promote penicillin synthesis by fungi lead to the fungi's death; the steroids, as well as numerous other microbial products, are toxic to the micro-organisms which produce them.

More than thirty years ago, V. L. Omelansky predicted a glorious future for bacterial chemistry: 'In the hands of the experimenter, the microbe becomes a docile instrument which performs biochemical conversions with the same certainty as that shown by the reagents currently used for chemical reactions'. The subsequent development of microbiology has confirmed this prediction. However, while playing their part as chemical agents, micro-organisms remain, none the less, living beings, with all their exacting requirements. We must know how to choose for them the particular cultural environment—a 'sanatorium' environment, so to speak—which corresponds to their physiological needs. In such conditions they are indeed the faithful servants of man, to whom they continuously yield new and indispensable products for medicine, for the chemical and food industries, and for agriculture.

An example of the interconversion of steroids by micro-organisms: from cortisone to prednisone.





This Petri dish allows observation of the inhibition of growth of B. roseus fluorescens *by various antibiotics: T3 tetracyclin, E6 erythromycin, C50 chloramphenicol, S60 streptomycin, 08 terramycin, P05 penicillin. Penicillin alone has no effect. (Photo Lod).*

Is aseptic life possible?

James A. REYNIERS

The question of whether higher animals can live in the absence of microbes was first raised by Pasteur (1885).[1] Although he did no experimental work on this problem and did not think life under such conditions would be possible, he was fully aware of the importance of such a study.

Over the years following Pasteur's remarks, many terms have been suggested to describe animals reared free from all life other than their own. The term generally used today is *germ-free*, which has taken on special connotations. Other terms which have been suggested and used occasionally are: 'aseptic' and 'axenic', 'gnotobiote', 'sterile', 'bacteria-free', 'pure'. In this chapter the term germ-free is used to describe the animal and also its conditions of existence. Time will probably demand a special terminology for this growing scientific field. One such term has been suggested, 'gnotobiotics' (*gnos*-known, *bios*-life, Gr.) since it is presumed that the kind of life being studied is experimentally defined and hence 'known'.

The question of whether germ-free life is possible for higher animals should be restated for completeness and clarity. It should be considered in terms of the pure-culture concept and include additional criteria which mark a successful species, ie, whether animals can live, breed, and pass into successive generations and so meet the biological demands of their environment. Thus, a colony of germ-free rats may be considered a pure culture of rats in the same sense that microbiologists speak of a pure culture of bacteria.

On the basis of the above-mentioned criteria, the answer to the question is a simple affirmative. Higher animals can live out their lives and breed generations in the absence of any detectable living contaminants, fulfilling the pure-culture concept and the biological criteria of adjustment to the environment.

Germ-free animals

Within the limits of present-day techniques for detecting contamination, a number of species of higher animals have been obtained germ-free and some of these species have been reared through many generations. For example, the rat and the mouse have been reared through more than twenty generations, the chicken and guinea-pig through two, and the Japanese quail (*Coturnix japonica*) through four. The rat and the mouse have survived for over 1,000 days (the limit of the experiment), the chicken, Japanese quail and guinea-pig for more than a year. Thus, for these species at least, the criteria of biological success have been met.

Many other species of animals have been obtained germ-free and reared for varying lengths of time. Among these are: monkeys, dogs, cats, rabbits, pigs, goats, sheep, hamsters, turkeys, and a wide variety of invertebrate metazoa such as flies, cockroaches, round worms, and snails. The invertebrates have all been cultured through their life cycles and through many generations. The vertebrates mentioned have not been bred through generations, but this is largely a matter of experimental technique. For example, it is easier to obtain numbers of germ-free chickens from eggs laid by normal birds than it is to remove their oviducts. Many of the other species have long generation times and are too large to be maintained in colonies.

Since it is apparent that animals can live out their lives in the absence of microbes and other contaminants, the question then turns on the effect of these foreign living beings on the animal. Here there has been a common tendency to compare germ-free animals to those reared normally.[2] This is not a valid comparison, for reasons which will be discussed.

In the early work with germ-free life a comparison with the normal counterpart was

(1) *Proceedings of the French Academy of Sciences, 5th January, 1885 (p. 58).*

(2) *The term normal is used to designate animals reared outside germ-free isolators, and implies contamination with an experimentally controlled variety of living contaminants such as bacteria, parasites, and viruses.*

Reyniers' isolation units are shown in this laboratory; they are used for rearing colonies of small mammals and birds. The technician is standing by a mobile steam generator with which the barriers of the isolation units are sterilized. With this method, these units can be in continuous service for several years without replacement of gloves or filters.

necessary simply because little was known of the germ-free animal *per se*. Times have changed and with the use of germ-free animals as experimental tools, the germ-free state is established and is no more comparable to the normal state than a pure culture of microbes is comparable to the total microbial flora in a given situation.

All animals of a species have the same anatomical and physiological potentials and the same evolutionary background whether they are reared germ-free or not. The difference lies in the fact that the normally reared animal is, from birth to death, the host for large numbers and many kinds of living contaminants. The germ-free animal is, on the other hand, at no time in contact with forms of life other than individuals of its own species living the same kind of existence.

Microbial contamination

All animals are naturally equipped with the necessary systems and defences to permit them to adapt to the environment into which they are born. Such systems and defences are prepared during embryonic development but may not be activated fully until called upon. In the ordinary way this contamination takes place at birth. In the germ-free state the animal is not in contact with living contaminants and it is reasonable to assume that such systems will remain idle, although present. The definition of these systems and their role in the economy of the animal can best be studied in the germ-free animal and in the germ-free animal experimentally contaminated with selected pure cultures.

Microbial contamination is an accident in the life of an animal. It is admittedly an accident with which the animal is prepared to cope, but this does not change the situation. The wide variety of species already obtained germ-free and the widespread use of embryos for virus growth and tissue culture, makes it clear that an animal is protected *in utero* or *in ovo* if for no other natural reason than that it must be so shielded in the early stages of development, since it has not had time to develop defensive systems of its own. It is this fact that makes it possible to obtain germ-free animals, since these animals are taken from the mother before birth or hatched in a sterile environment from a surface-sterilized egg.

There is always the question of whether microbes, parasites or viruses can penetrate the maternal barriers or are carried in the germ cells of the parents. The implication is that if this were so it would be impossible to obtain germ-free animals. The question is legitimate; the implication is not. The evidence that animals can be reared germ-free for many generations is by now firmly established, always, of course, within the limitation of the techniques and methods employed to detect contamination. On the other hand, there is equally good evidence that maternal barriers can be penetrated and that some animals are contaminated during their embryonic life. The point is that such animals are not used in germ-free studies, although they form an interesting field of study which needs careful consideration under germ-free conditions.

A germ-free animal can be obtained from a healthy mother and it is here that the inquiry starts. It is the common experience of those working with germ-free animals that contamination before birth is the exception rather than the rule. This may be due, in part, to the fact that only healthy, clean normal stock is used.

The problem of defining a living contaminant opens up a new area of thought when certain borderlines are approached. It is reasonable to assume that adequate tests are available for detecting bacteria, yeasts, fungi, ecto- and endo-parasites, protozoa and many viruses. Detection is made easier within the confines of a germ-free system by the fact that the animal itself is an excellent culture medium; and since it is kept in an environment to which no external or unwanted contaminant is added and observed for generations in this environment, the chances of detecting injury to the animal or growth of the contaminant become more certain.

Aseptic life is possible

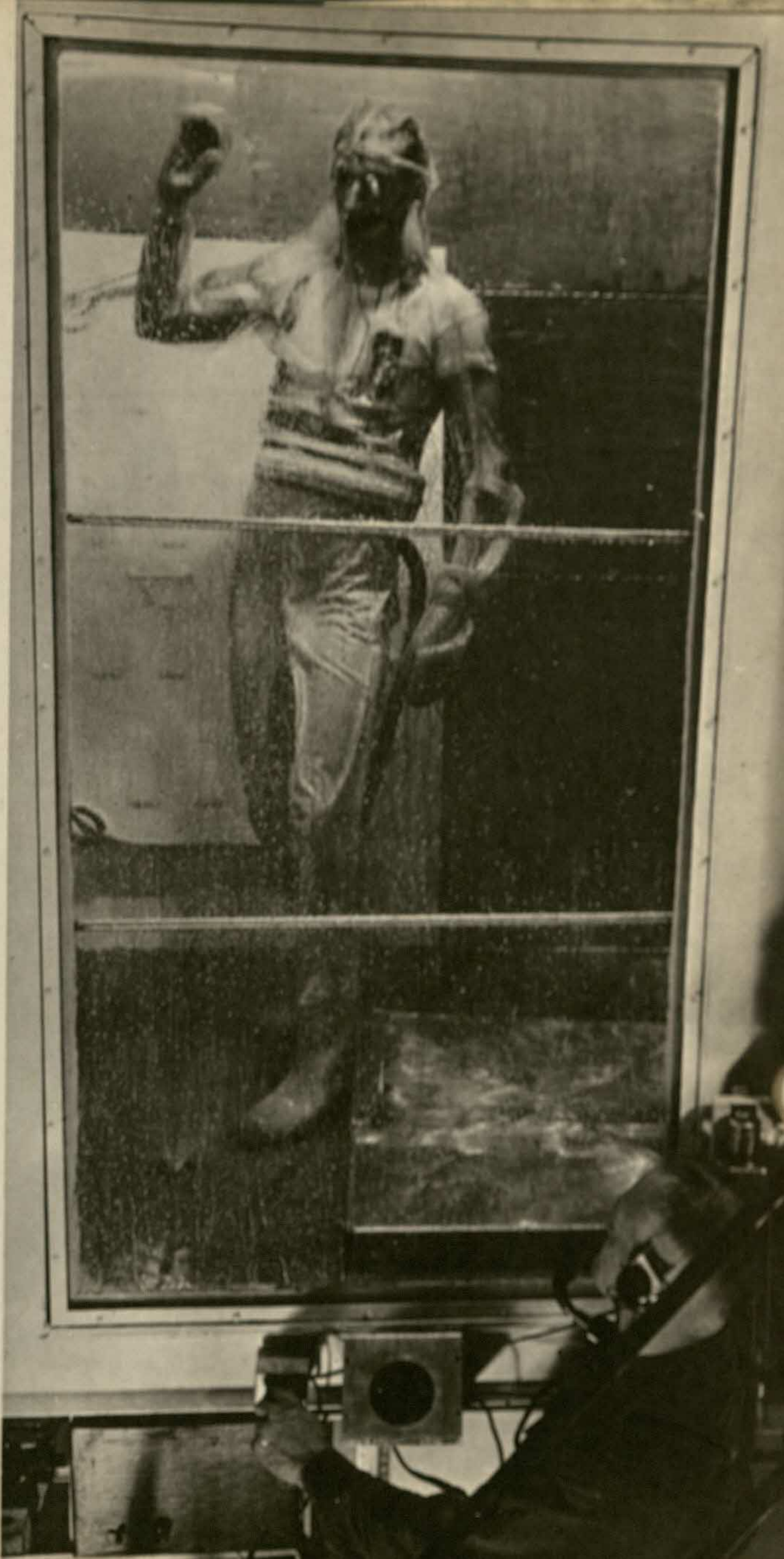
As in any new and exciting scientific field, there is always the possibility of thinking out new tests for contamination. For example, the problem of cancer-causing viruses is so closely tied to the genetic mechanism that it is difficult to name or even define them. Whether such viruses fit within the limits of present testing criteria constitutes a most rewarding field of study. It should be pointed out, however, that even if animals, recognized by one set of criteria as germ-free, are shown by another set of criteria to harbour viruses, it is reasonable to assume that not all animals of a given species will be so infected. Thus, the search would continue, by selection of animals in which the newly found virus is absent. The new tests for contamination would then be added to those already in use.

The point in all this is that today, within the limitations mentioned, it is reasonable to say that germ-free life is not only possible but can continue through generations. Contamination, therefore, should be regarded as an accident in the life of an animal, even though it is a common accident against which the animal has been prepared genetically and by maternal protection.

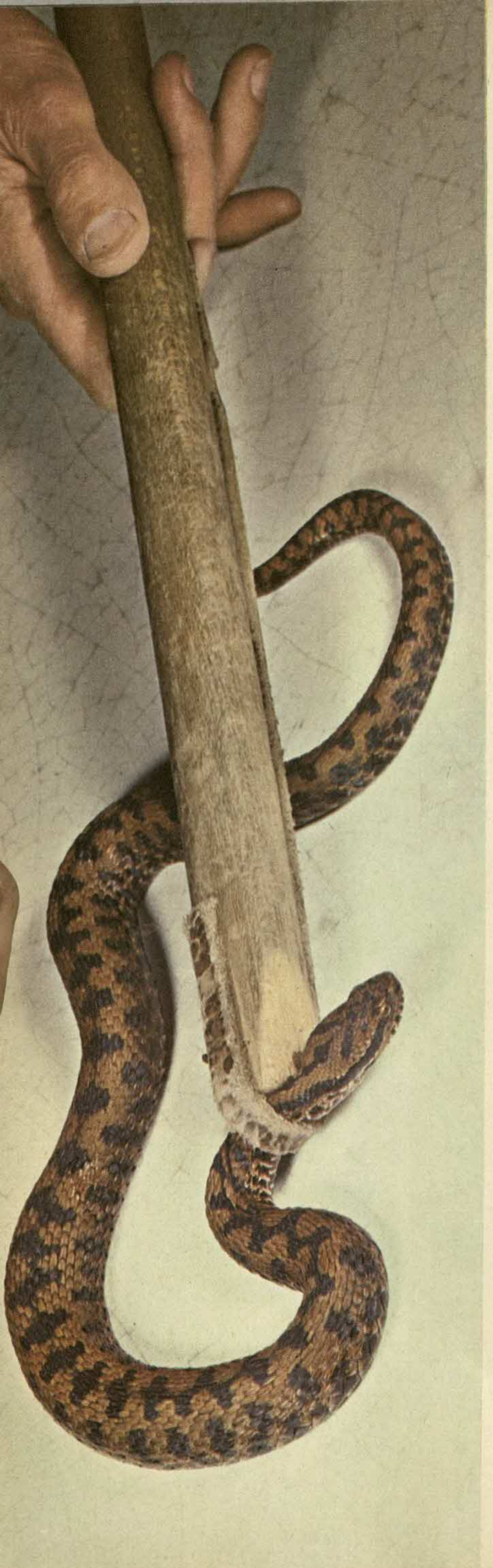
Methods of rearing germ-free animals

The rearing and using of germ-free animals experimentally entirely depends upon a specialized apparatus called an isolator. All isolators, regardless of design, include the following features: an impermeable skin entirely surrounding the contents; the property of being completely sterilized initially; the introduction of food and supplies which have been sterilized; ventilation with sterile air or a mixture of gases; clear visibility of the contents; transfer of the contents without contamination from one isolator to another; removal of waste and debris without breaking the barriers; a means of handling the animals; security for long periods of use without contamination. To these may be added convenience, flexibility to permit adaptation to experimental demands; specialization for unusual needs; adaptation to laboratory routine, and variation in size. It might be added that the cost of germ-free operations lies not in the initial cost of the apparatus but in failure to prevent contamination. Therefore, it is essential that whatever the apparatus used, it must, first of all, be capable of maintaining sterility throughout the term of an experiment and be large enough to rear colonies of small animals.

The germ-free isolator may vary from a simple glass jar or a plastic envelope to more highly specialized stainless steel enclosures of



Large-scale isolation units have recently been perfected for rearing large animals or for carrying out extensive trials. The operator can enter the isolation chamber, clothed in sealed plastic, with an air-supply. This suit is sterilized with detergents and bactericides or dipped in a tank containing germicide, as shown in this photograph.



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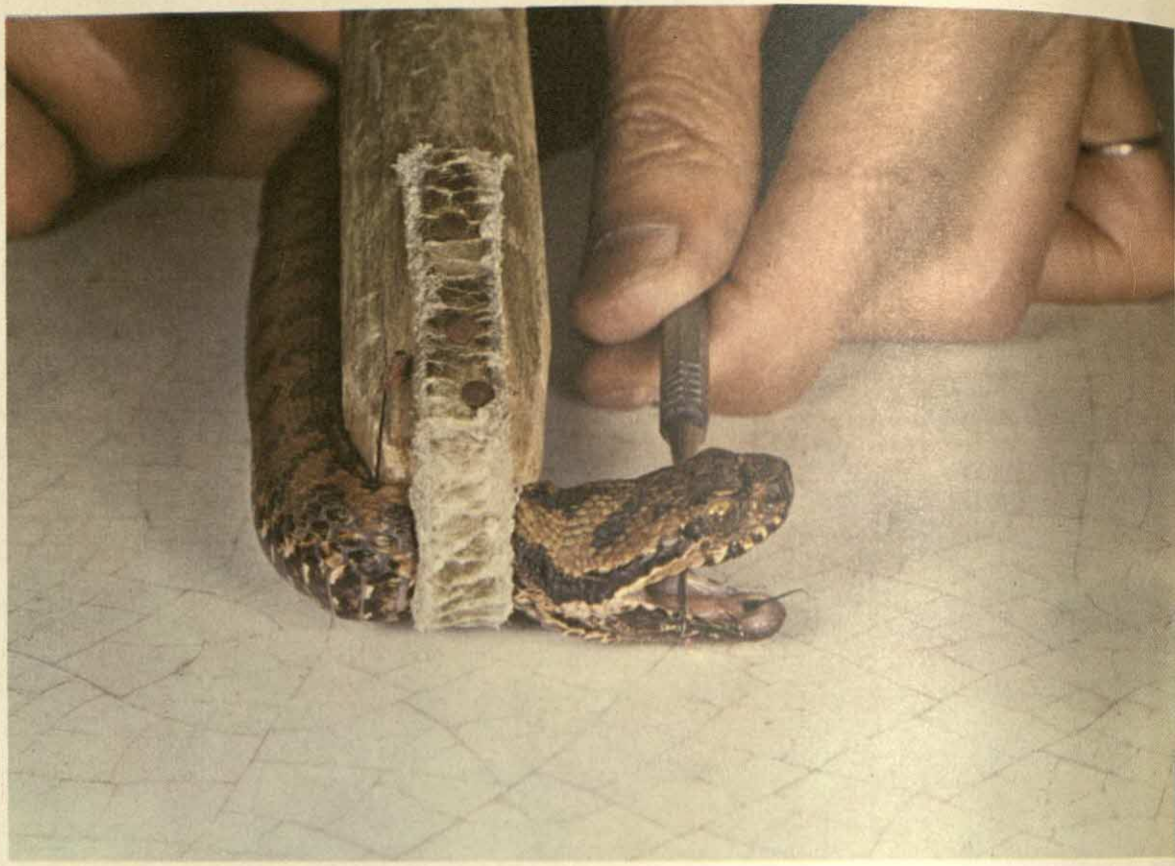
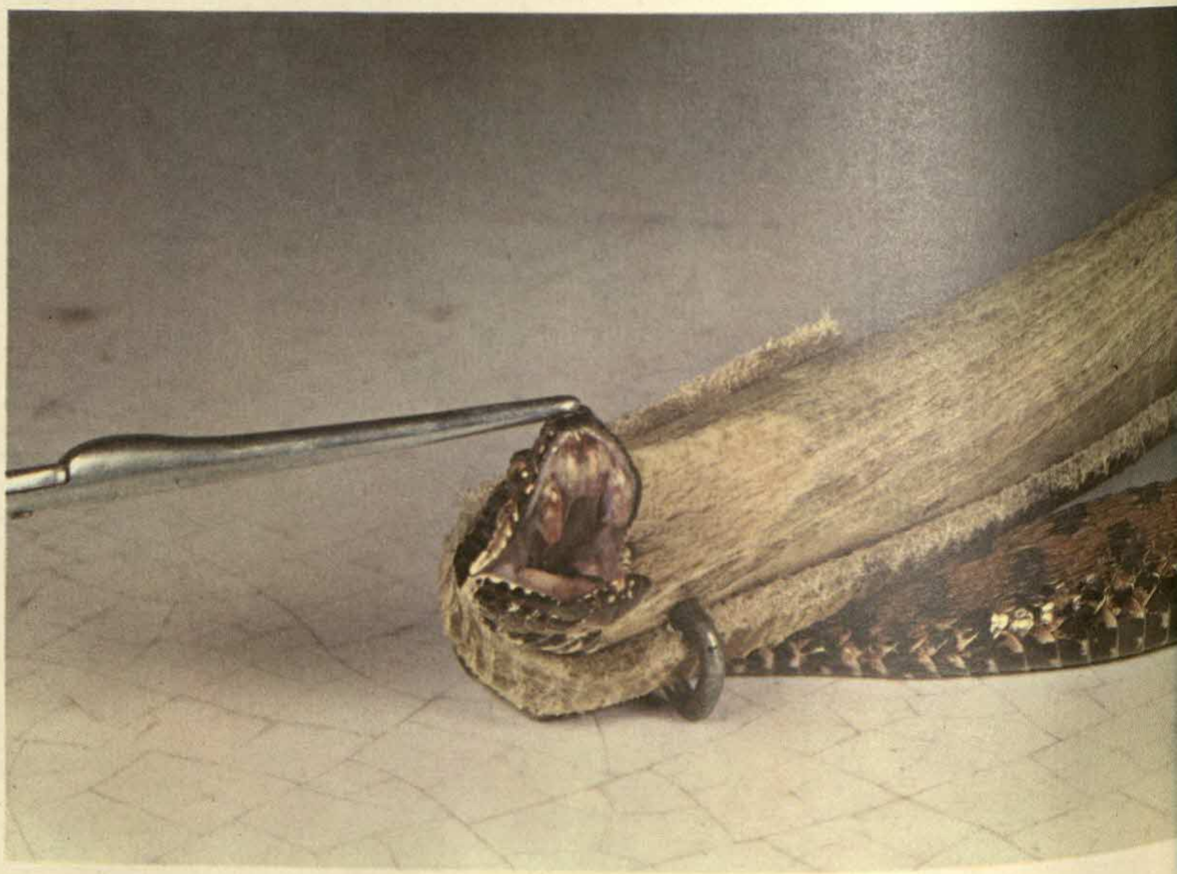


In order to manufacture antivenom sera, horses are vaccinated with snake venom. Here (1) the viper's neck is caught in a stick and strap and (2 and 3) the jaws of the viper are forced open.



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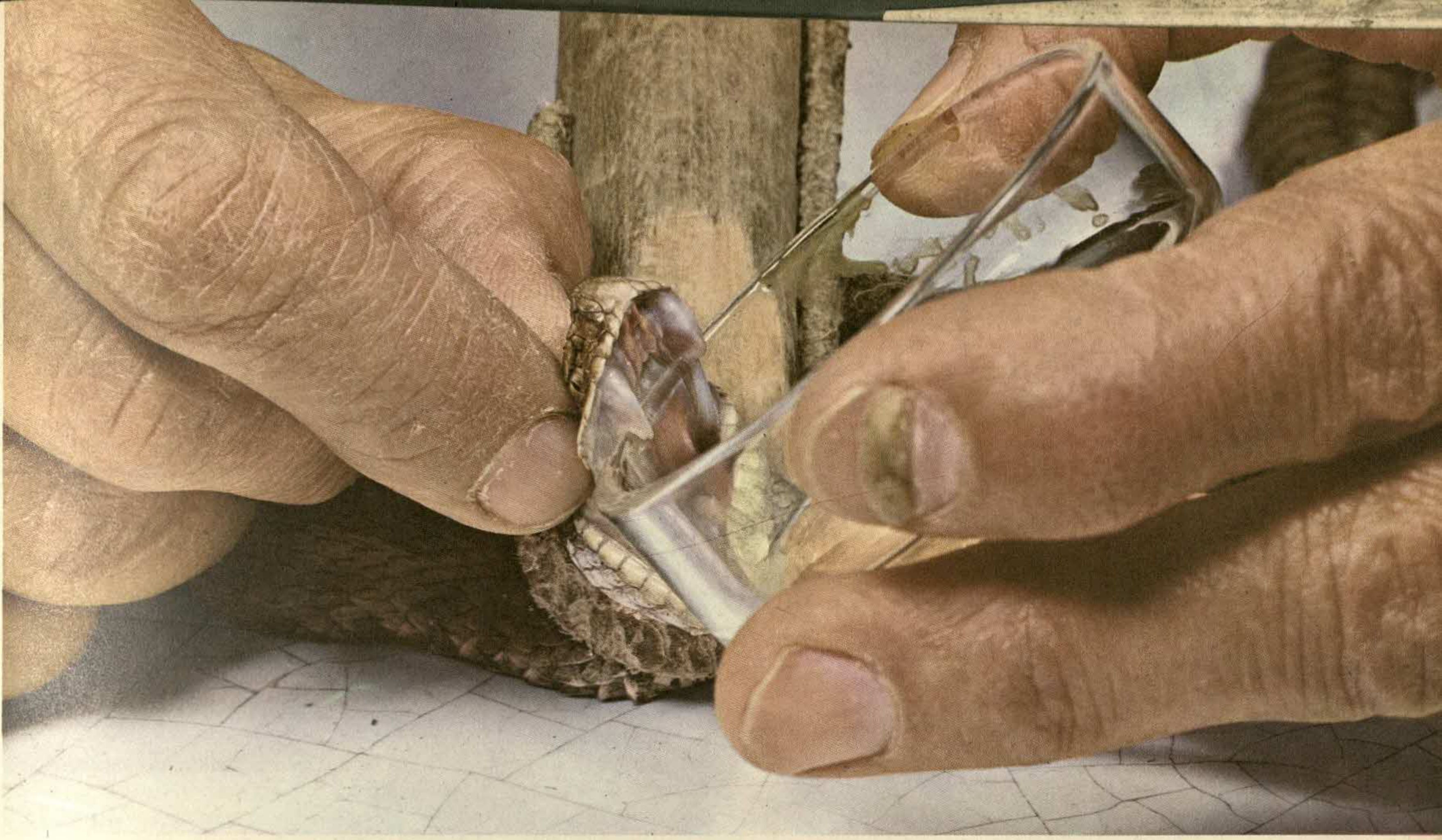

The venom drips from the end of the viper's fangs. The operator collects it (4) in a small glass dish. Here we see (5, 6) two types of dried venom; white and yellow venom. (7) Immuno-electrophoretic examination of Vipera aspis *venom. The curved lines indicate the points of combination between the antigens of the venom and the corresponding antibody. (Photo Lod. Pasteur Institute, Garches).*

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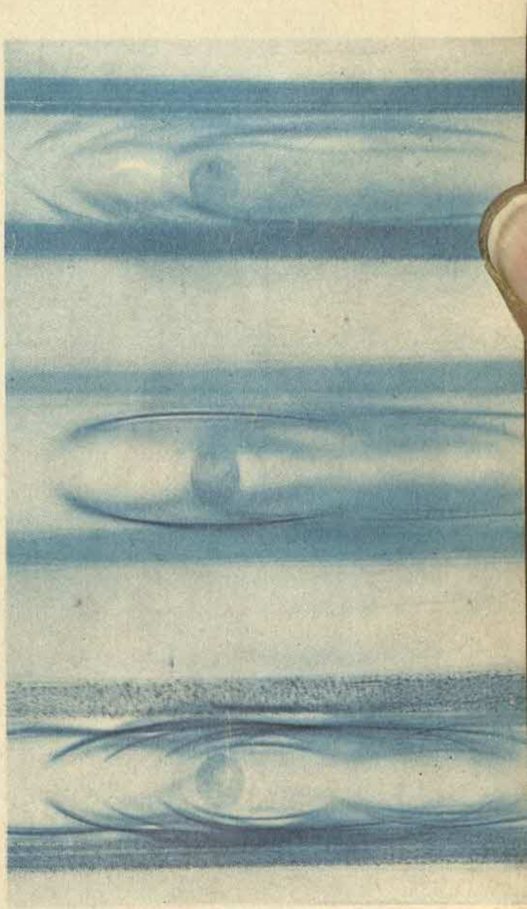

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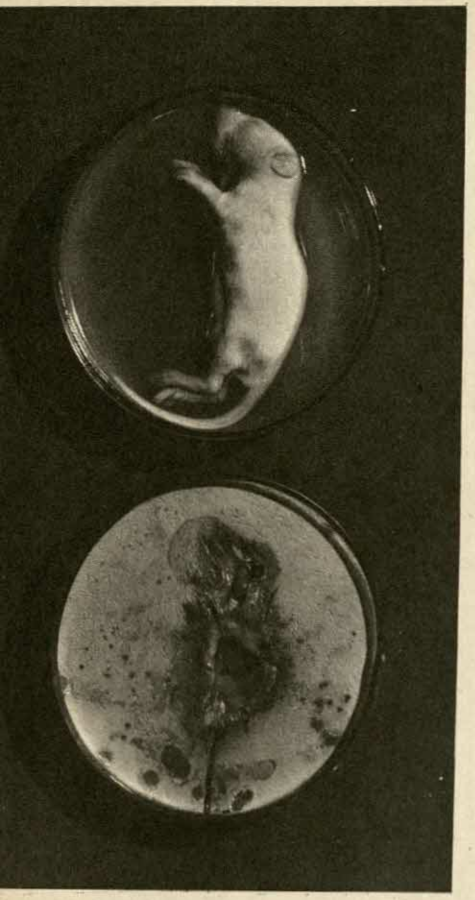


A microbe-free rat (above) compared with a normal rat (below) after three days' incubation in an agar medium.

HISTORICAL NOTES

1885
L. PASTEUR communicates E. DUCLAUX'S work on the culture of peas and beans in a sterile medium, to the French Academy of Sciences, directing attention to the question of whether higher animals can live in the absence of microbes.

1886
M. NENCKI, in Switzerland, challenges the Pasteur-Duclaux thesis.

1895
G. H. F. NUTTAL and H. THIERFELDER in Germany, perform the first experiment on higher animals, using guinea-pigs and chickens.

various shapes. For example, it is possible to rear germ-free chickens from eggs hatched in a sterilized glass jar which contains a supply of food and water, or to maintain animals germ-free for short periods of time in simple envelopes or boxes. Such apparatus is limited but useful.

The Reyniers germ-free system is the most widely used at present. It consists of a basic isolator usually cylindrical in shape and made of stainless steel. To this are attached the necessary air filters, rubber gloves, sterile seal and windows. It is made to be sterilized *in situ* with steam under pressure. This basic isolator has been modified into the following specialized isolators: surgical, transfer, connecting and rearing. The basic design has been further modified for use in physics and chemistry, where assembly, machining and testing must be done in an artificial atmosphere free from oxygen. The isolators can be combined into multiple systems in which many individuals may work simultaneously on an experiment. Food and other supplies are autoclaved into the isolator via the attached double-door seal. Hysterectomies are performed in the surgical isolator, which is attached to a rearing isolator by the sterile seals. The young animals are then placed in the rearing isolator where they are hand-fed to weaning, after which they breed and care for their own young. Eggs with the shells sterilized are incubated in the isolator till they hatch. Germ-free animals can be transported in simple glass jars and returned to a germ-free system through a transfer isolator. This makes it possible to grow the animals in a central laboratory and to transport them to smaller establishments designed for using but not producing colonies.

An isolator has been built for purposes of rearing large animals, maintaining large colonies of smaller animals and performing large-scale experiments. The attendant can enter this isolator room clothed completely in a plastic suit, supplied with air for breathing. The surface of the suit is sterilized by a series of detergent-germicide showers, or by complete submersion in a tank of germicide which also acts as a sterile seal. Animals may be put into or removed from this system in the standard germ-free isolators attached to it.

There are many specialized problems involved in the design and operation of germ-free isolators which cannot be fully discussed here; for example, the problem of providing a supply of air completely free from contaminants and of specified humidity, temperature, and pressure. There is also the problem of sterilizing food. The most certain method of accomplishing this is by autoclaving it, and for special purposes this can also be accomplished by radiation and by the use of germicidal gases. The main essentials in all these problems are that absolute sterility be attained, that the isolator should operate for long periods without contamination, and that the operations be accomplished with precision and certainty, according to easily followed routines.

All viviparous animals are obtained by Caesarian section, usually performed in a specialized surgical isolator by operating on a timed pregnant mother, using a cautery. All oviparous animals are obtained by first sterilizing the surface of the fertile egg with a germicide and introducing the eggs into an isolator through an attached germicidal trap, where they are incubated until they hatch.

The Caesarean-born young must be fed by hand on an artificial diet previously autoclaved. The guinea-pig is an exception to this, since it will feed itself with a semi-solid diet. Birds and other oviparous vertebrates hatched into a germ-free isolator will feed themselves after hatching. The mammals, on weaning, continue to feed themselves and when they give birth to young will feed and care for them. Closely connected with feeding Caesarean-born mammals are the important and, as yet, little understood nutritional and physiological requirements of the suckling animal. These can best be studied in the germ-free animal, either by hand-feeding selected diets or by studying mother-suckled young.

Characteristics of germ-free animals

It is only possible here to deal in generalities, partly because of space and partly because the germ-free animal is relatively new to science and has not yet been studied to any great degree.

Germ-free animals look and behave like normal animals. They grow well and live for long periods of time. Perhaps the most noticeable characteristic is the lack of faecal odours or the smell of ammonia usually associated with normal animals. Another somewhat remarkable difference follows the death of a germ-free animal. There are no odours of decay and, indeed, the animal changes little in gross external appearance. Post-mortem changes present an interesting field of investigation which has as yet to be carried out systematically. In the germ-free animal kept in a moist and warm environment following death, there is some enzymatic dissolution of tissues, but this is confined rather markedly to the organ or system and is not as general as with the normal animal, where final dissolution is aided by microbes. An enzyme map made after the death of the germ-free animal should be of value, as would a study of tissue and cellular death.

It is interesting to consider the taste of the flesh of germ-free animals, such as chickens. It has no more savour than smell. It should be pointed out that the change in odour and flavour following contamination of a germ-free animal may well have important practical implications.

In general, the intestinal tract of germ-free animals is thinner and has a greatly reduced lymphatic content, less musculature, and is paler in colour. Yet this tract is fully functional as far as digestion is concerned and serves the animal well for growth and activity. It suggests that in the germ-free animal the intestinal tract is 'stripped to the essential elements' (Gordon) and is largely an organ of digestion, whereas in the normal animal it serves an additional purpose, that of containing the microbial flora. This is further borne out by studies concerning lymphocytes, the supply of various segments of the intestinal tract and penetration of microbes through the intestinal wall. In the germ-free animal there is a distinct suggestion that the lymphatic system is idling and that the intestinal wall is quickly penetrated by bacteria.

One of the conspicuous differences is the greatly increased size of the caecum in germ-free animals such as rats, mice, guinea-pigs, and rabbits. The caecum of the adult germ-free guinea-pig may constitute one-third of the total body weight of the animal. Yet the animals grow and breed with the enlarged caecum and attempts to remove the caecum have added little to the function and well-being of the animal.

Organs and systems not ordinarily in direct contact with microbes, eg, the heart, thymus, spleen, and endocrine glands, are similar in size and weight in both the germ-free and the normal animal. The erythrocyte and haemoglobin content of the blood show little difference, whereas the absolute count of circulating lymphocytes is lower in germ-free animals. It is also interesting to note that those organs usually in direct contact with microbial flora, such as the intestinal tract, show a lower coefficient of variability with respect to weight in the germ-free animal. This suggests that the germ-free state is one to which the animal has adjusted itself and that there is a lesser environmental demand.

From the biochemical point of view, the intestinal contents of germ-free animals and the contents of tissues and organs are not significantly different when the animals are on a complete diet. There is no indol or skatol in the intestinal contents. The intestinal contents are composed of amino acids, vitamins and other adjuvants, making the contents an excellent microbiological culture medium. Vitamin deficiencies can be induced in germ-free animals with a difference in balance but not in kind.

Serologically, the germ-free animal shows natural antibodies to foreign red blood cells and sera, but unless microbes are present, either living or dead, in the diet, antibody production is lacking. This can be induced in germ-free animals in the usual manner with selected antigens. Phagocytic activity against bacteria, particularly that of circulating or wandering phagocytes, is lacking in germ-free animals. Again, this can be stimulated either by feeding or injecting microbial antigens. This suggests that the antibody-forming apparatus is present but is inactive in the germ-free animal, but that it can be stimulated or activated.

As far as nutrition of germ-free animals is concerned, they have similar qualitative requirements to normal animals. All diets fed to germ-free animals must be sterilized, usually by autoclaving, but these diets are calculated from experience to contain all the necessary factors following sterilization. So far as can be determined in the chicken, no new vitamins could be found in liver and yeast which are necessary for germ-free life. The point is, simply, that the germ-free animal fed on a complete diet grows comparably with, or slightly better than, the normal animal, reproduces as well, and rears its young without difficulty.

The above attempt to characterize the germ-free animal is general. A deeper systematic study, which has been done only for the chicken, is needed before differences in detail and for each species can be described. This will be necessary in the future, if for no other reason than that of getting to know thoroughly the germ-free animal used in experimental biology.

Uses in experimental biology

The germ-free animal is used in the same way and to the same extent as any sterilized

1899
M. SCHOTTELIUS, working with guinea-pigs, supports Pasteur's view that microbes are needed for higher animals.

1901
E. METCHNIKOFF (France) supports Nencki's thesis.

1912
M. COHENDY (France), working with chickens and later (1914) with guinea-pigs, was the first to use germ-free animals experimentally in a study of bacterial infection.

1912
E. KUSTER (Germany) reared two germ-free goats. By now the original thesis of Pasteur had been negated to a great extent but not refuted experimentally.

1928
J. A. REYNIERS (U.S.A.) started a long-range systematic programme for the study of germ-free life, based on extending the pure-culture concept in experimental biology. The aims of this programme were realized thirty years later in (1) the development of a dependable mechanical system of germ-free isolators; (2) the development of methods for obtaining and rearing germ-free animals as pure cultures; (3) the study and description of a wide variety of germ-free vertebrates; (4) exploration of the usefulness of the germ-free animal and techniques at the theoretical and applied levels, and (5) establishing the first research institute devoted to germ-free life studies.

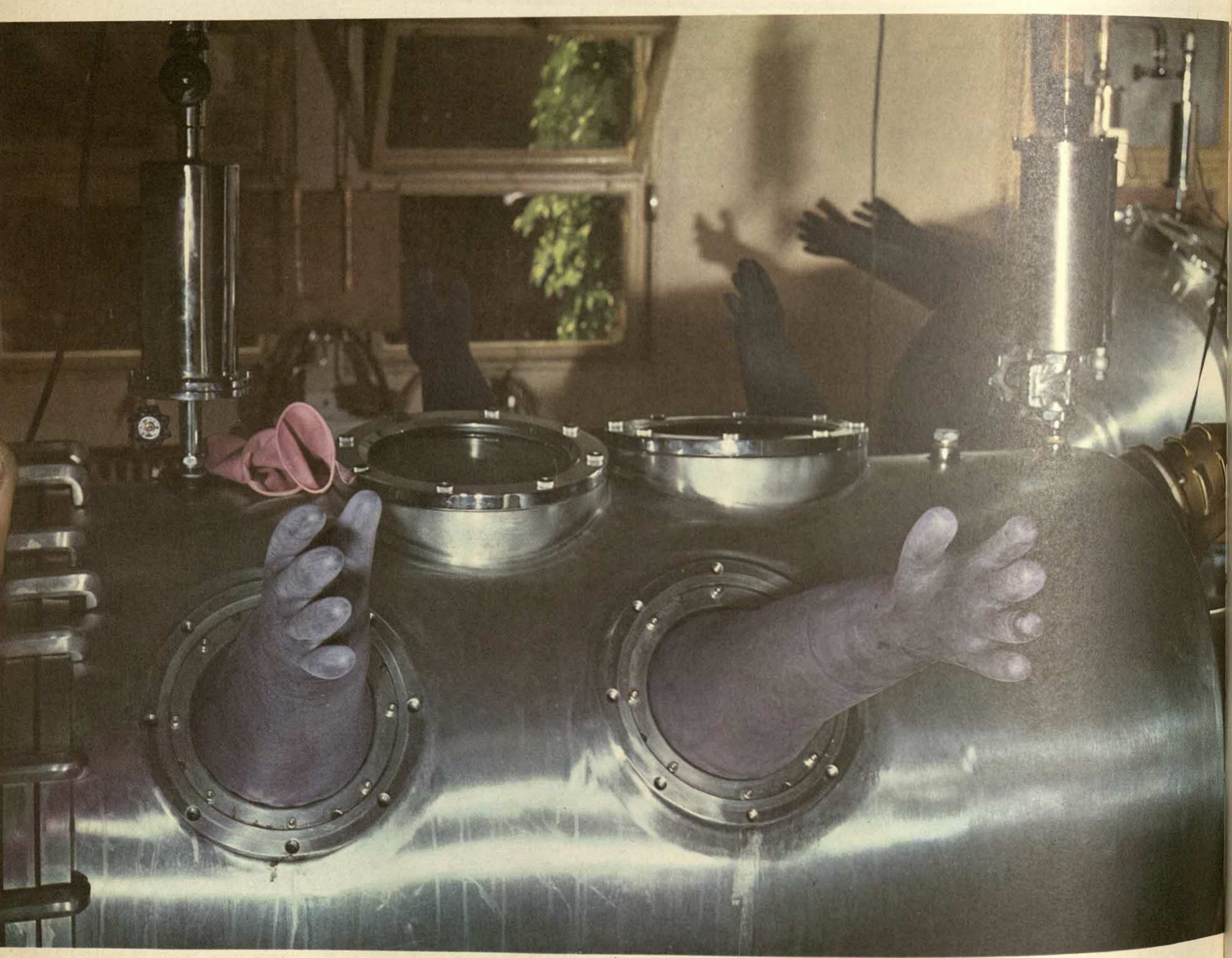
1932
G. GLIMSTEDT (Sweden) used the germ-free guinea-pig in a histological study of the reactions of secondary lymph centres.

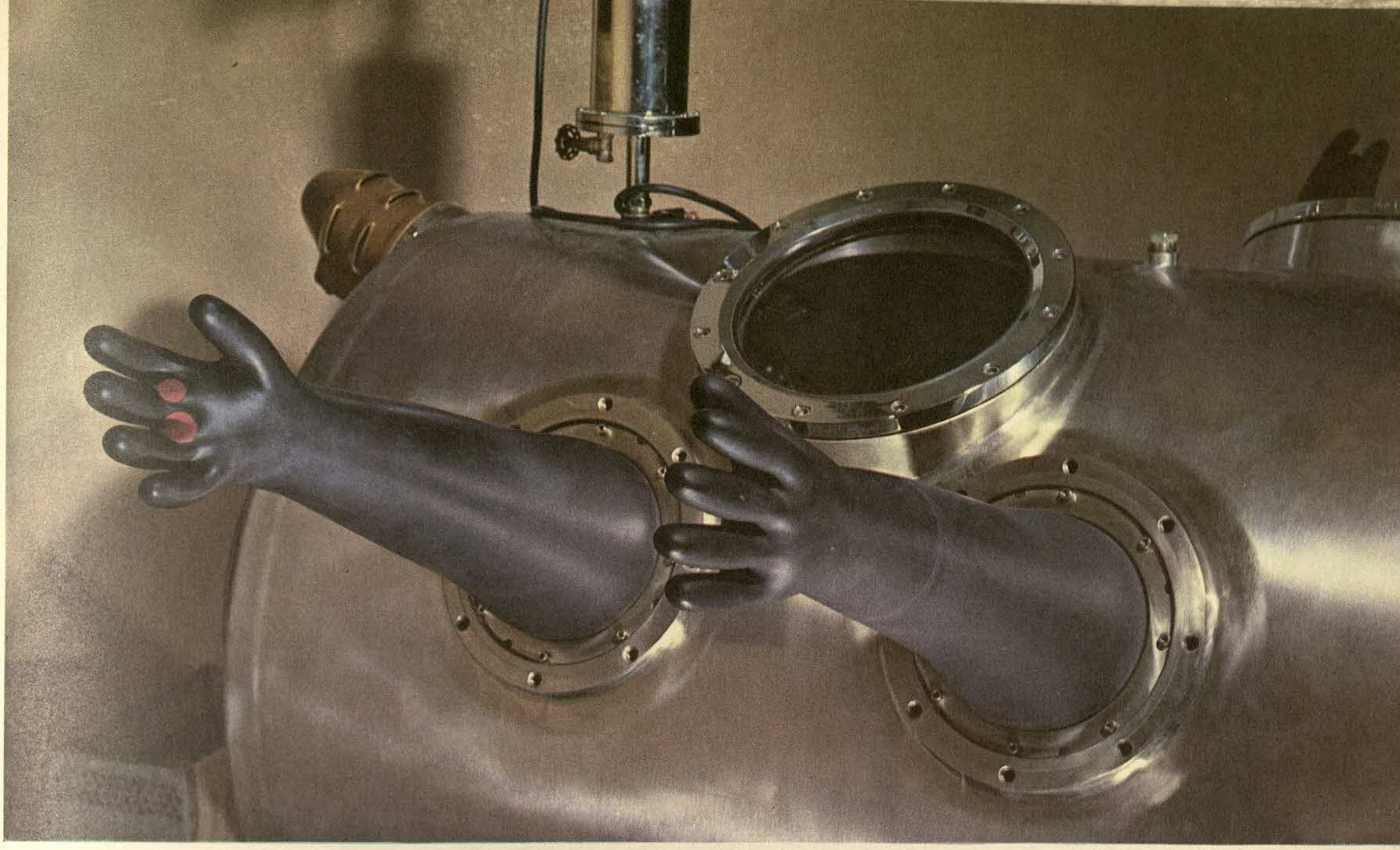
1937
N. BALZAM (France) used germ-free chickens for the study of vitamin metabolism.

1939
First symposium on microsurgery and germ-free methods held at the University of Notre Dame, Notre Dame, Indiana, U.S.A.

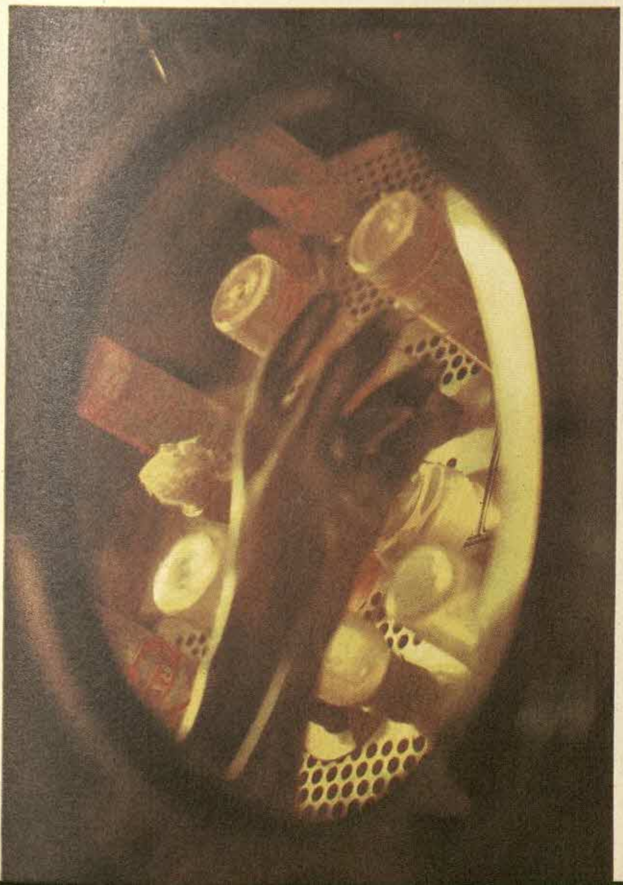
Aseptic animals

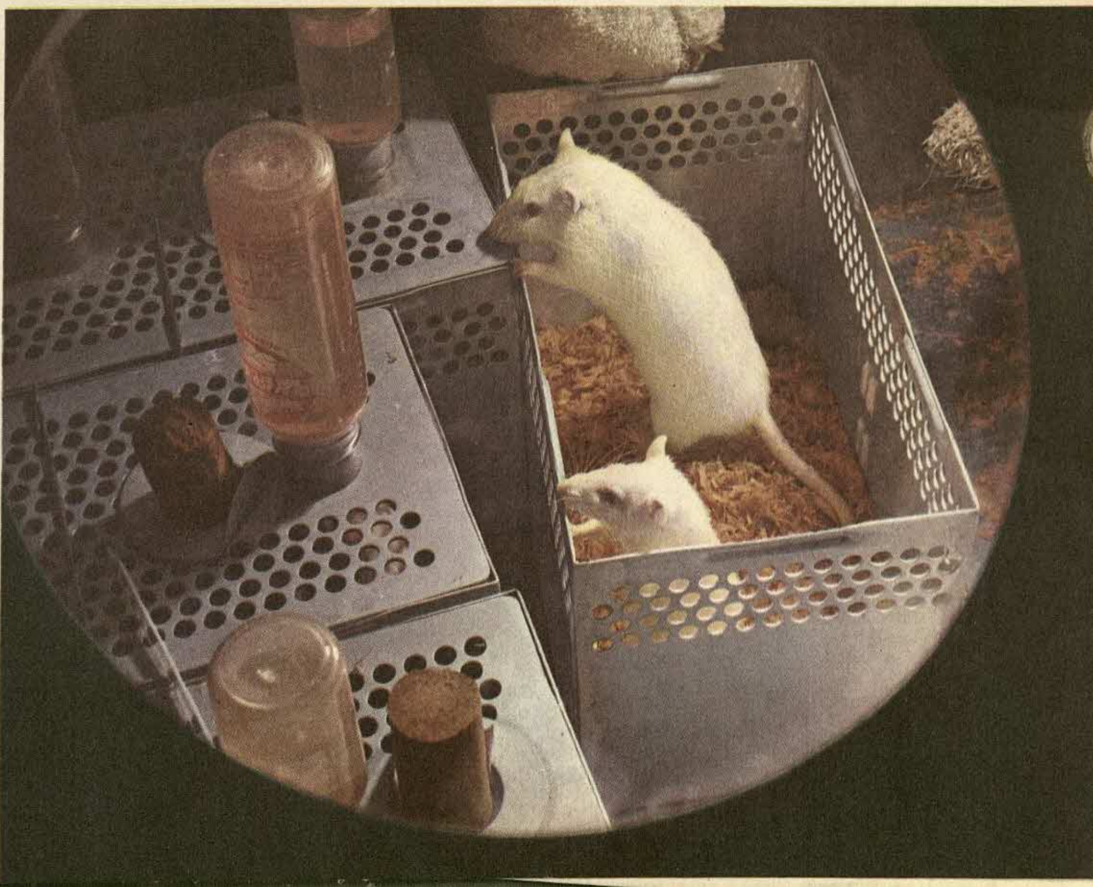
The rearing and experimental use of aseptic animals depends on the use of isolators with certain fixed characteristics: hermetic sealing, and means of observation, manipulation and disposal of waste. The Reyniers system is shown here—a cylinder isolator of stainless steel, to which are fitted an air filter, rubber gloves, hermetic sealing and observation port-holes.





Here is the latest model of an isolator, intended for rearing small mammals. The white mice, photographed through the port-hole, live in grilled cages, and their food is carefully sterilized. The food requirements of the aseptic animals are qualitatively the same as those of normal animals. If certain deficiencies are provoked in vitamins B_1 *and K, severe forms of anaemia are caused. But rats subjected to a diet encouraging dental caries show neither microcaries nor macrocaries. Such experiments are of interest for the use of aseptic subjects in biology and experimental medicine. (Photo Lod, by courtesy of M. Sabourdy, Centre National de la Recherche Scientifique).*





1947
Work on germ-free life resumed in Sweden by B. GUSTAFSSON, *using germ-free rats and in Japan (guinea-pigs) by* M. MIYAKAWA.

1958
Symposia on germ-free invertebrate metazoa and germ-free vertebrates held at New York Academy of Sciences, New York, N.Y., U.S.A. Symposia on germ-free life held at the seventh International Congress of Microbiology, Stockholm. First shipments of germ-free animals by public transport, thus making widespread research possible.

1959 to date.
Establishment of many germ-free life research centres in the U.S.A., France, Japan, Sweden, and more extensive use and study of germ-free life with many scientific disciplines. The germ-free animal is no longer a scientific curiosity but a valuable and necessary tool to modern science.

medium which will support the life under consideration. There is, however, one important difference. The germ-free animal is a living medium with all the responses of a living animal to changed conditions.

It is possible to study the germ-free animal *per se* as a unique phenomenon in the world, with all that this implies as to nutrition, defensive mechanisms, ageing, or any other systemic development. In this way base lines can be defined for comparison with any given situation experimentally specified.

Once the germ-free animal is known better, it is possible to add a pure culture to it, or to add any number of pure cultures to bring it to a desired ecological level.

In addition to their usefulness in connection with germ-free animals, the isolators and techniques can also be used to contain dangerous pathogens, as in aerobiological studies, or for various chemical and physical experiments where the physical environment must be controlled.

In this short article only a few of the many problems in which germ-free animals have already been used can be mentioned. The examples represent types of experiment.

Nutrition: the intestinal synthesis of vitamins and their use by the animal remains a perplexing problem. Several aspects, particularly those of the effects of Vitamin B_1 and K have been studied. A severe hypoprothrombinaemia can be produced in germ-free rats on a diet in which vitamin K is absent. These symptoms can be alleviated if the germ-free animal showing the deficiency is exposed to contamination or given K orally. The kinds of micro-organism concerned and the exact physiology remain to be determined.

Dental research: the etiology of dental caries has been studied. Conventional rats on an autoclaved cariogenic diet all show dental caries. The same diet fed to germ-free rats causes no caries, either microscopic or macroscopic. The production of dental caries is either very low or absent when the animals are contaminated with a pure culture of acidogenic bacteria, but caries can be produced with pure cultures of enterococci or streptococci.

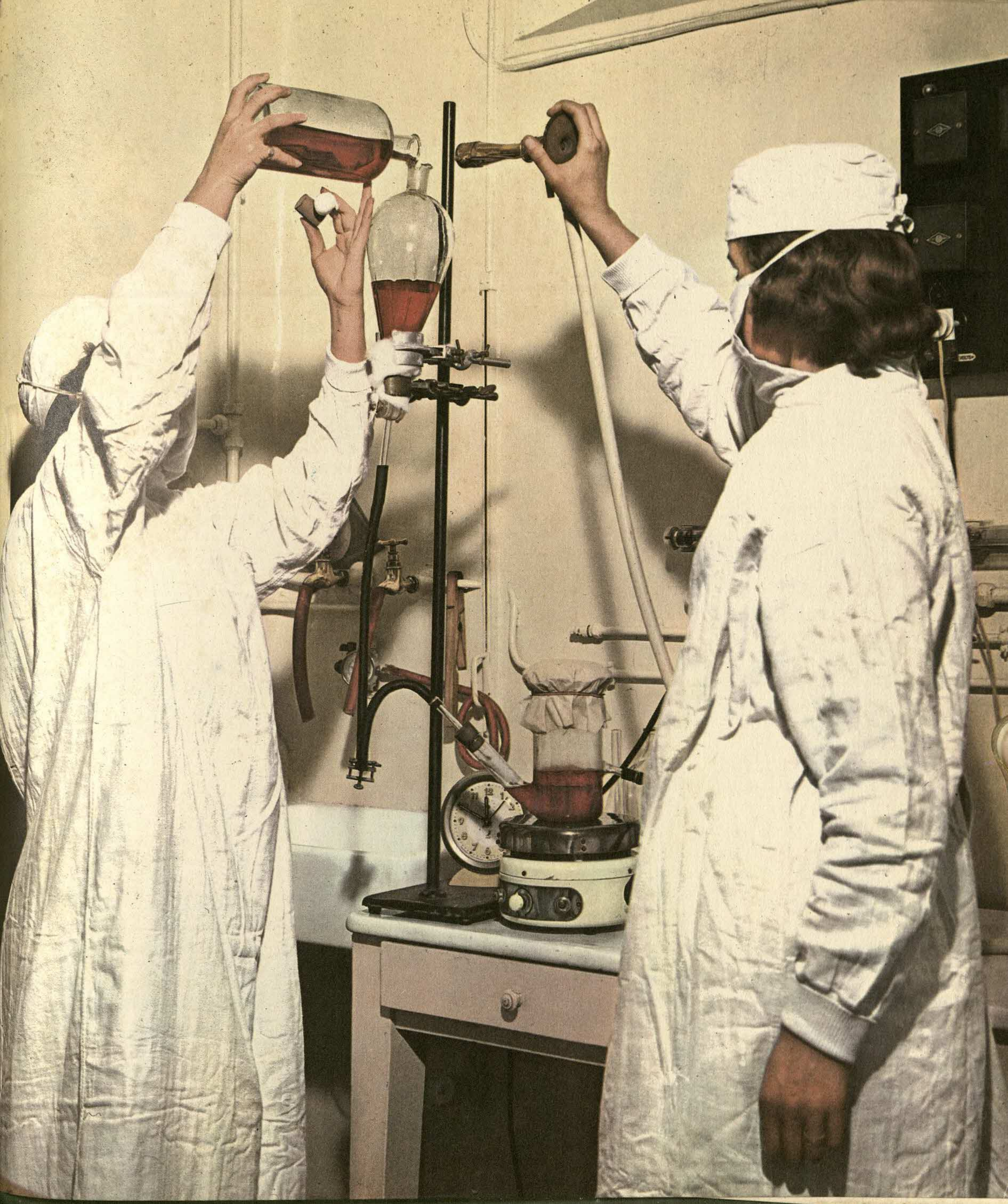
Microbial associations: amoebic dysentery is associated with *Entamoebia histolytica* and bacteria. The question is whether the amoeba alone can cause the lesions or whether it must be associated with micro-organisms, and if so, which kind. In a series of experiments, it has been shown that pure cultures of *E. histolytica* inoculated intracaecally into normal guinea-pigs will propagate and cause lesions and death. No lesions or long survival of the amoeba can be demonstrated in germ-free guinea-pigs. When the germ-free animals are given pure cultures, particularly of coliform bacteria, the lesions will form and the amoeba will propogate.

Cancer research: this is a relatively new field for germ-free life studies but it is particularly aimed at a viral etiology. Mammary cancers are regularly produced in female C_3H mice. In a series of experiments it was indicated that these are not produced in germ-free mice kept for 1,000 days. In chickens, fibrosarcomas produced by injection of methylcholanthrene can be transmitted readily from germ-free bird to germ-free bird with a shortened time to the appearance of the tumours following successive transfers. This type of tumour in normal birds cannot be transmitted by cellular passage. The tumour from germ-free birds, on the other hand, can be transmitted to normal birds and the passages continued in normal chickens. The fibrosarcoma from germ-free birds has been broken down into subcellular particles and the larger particles will cause the tumour, but smaller particles cause a severe wasting also, which can be transmitted as such from bird to bird.

In giving the above examples of usefulness, it is evident that there are many other fields in which germ-free animals have been and are being used. Their usefulness in virology has been indicated, as has their use in studies dealing with antibody and defence mechanisms. There are a number of diseases, such as liver necrosis, arthritis, dermatitis, allergy, and infectious complexes, which are being studied with the use of the animals and the techniques. The availability of germ-free animals and the apparatuses which can be used with certainty is relatively recent. Many centres for studying and using germ-free life have been established in the world and these are increasing. As experimental biology moves towards more exact demands the germ-free animal becomes more necessary.

Many years have passed since Pasteur first enunciated the problem of germ-free life. Today, there can no longer be any question about whether higher animals can live germ-free. Attention will now turn to studying life under these conditions and the use of germ-free animals as a tool in experimental biology and medicine. As the inquiry is pushed to theoretical limits concerning contaminants, it can well be expected that new frontiers will open.

Laboratory work with aseptic media: technicians preparing a tissue culture. (Photo Lod, Pasteur Institute, Garches).



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